PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION** see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
P92787WO		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/GB 01/00241	22/01/2001	20/01/2000
Applicant		
RES-DEL INTERNATIONAL LIMITED		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readble form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. _____

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [X] None of the figures.

INTERNATIONAL SEARCH REPORT

[redacted]ational Application No
PCT/GB 01/00241

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A01N1/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, PAJ, WPI Data, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 98 04127 A (STANKO RONALD T) 5 February 1998 (1998-02-05) cited in the application the whole document page 9 ---	1-14
A	EP 0 627 161 A (SIGMA TAU IND FARMACEUTI) 7 December 1994 (1994-12-07) the whole document ---	1-14
A	US 5 110 722 A (ANDEREGG KATHERINE A ET AL) 5 May 1992 (1992-05-05) the whole document table II --- -/--	1-14

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 May 2001	22/05/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Bertrand, F

1

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	DATABASE WPI Week 199251 Derwent Publications Ltd., London, GB; AN 1992-420365 XP002167149 & JP 04 316483 A (NIPPON SUISAN KAISHA LTD AND NISSUI PHARM CO LTD), 6 November 1992 (1992-11-06) abstract -----	1-14

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 01/00241

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 9804127	A	05-02-1998	NONE		
EP 0627161	A	07-12-1994	IT	1261695 B	29-05-1996
			AT	170709 T	15-09-1998
			DE	69413120 D	15-10-1998
			DE	69413120 T	11-02-1999
			DK	627161 T	07-06-1999
			ES	2122214 T	16-12-1998
			JP	7048329 A	21-02-1995
			US	RE36331 E	05-10-1999
			US	5496821 A	05-03-1996
US 5110722	A	05-05-1992	AU	6759690 A	13-06-1991
			WO	9107086 A	30-05-1991
JP 4316483	A	06-11-1992	NONE		

PROCEEDINGS
OF THE PHYSIOLOGICAL SOCIETY
OF NEW ZEALAND

IN VITRO BIOASSAY TECHNIQUES - QUESTIONABLE VALIDITY USING VERTICAL BATH TECHNOLOGY AND PHOSPHATE BUFFERED SOLUTIONS

D Rees, School of Biological Sciences, Victoria University of Wellington.

The ability of a physiological solution to sustain the overall metabolic function of isolated tissue/organ preparations, over an experimental period, should be a basic requisite for any pharmacological bioassay test regime, if validation of the observed physiological responsiveness to planned or random drug dose regimes is to be meaningful. The formulation of Res-Del® RS-I Mammalian solution, in tandem with the advanced perfusion technology provided by the *horizontally, aligned* Res-Del® Perfusion Bath, has been shown to maintain the physiological viability and pharmacological responsiveness of isolated mammalian tissues/organs at normothermic temperatures and for up to 12 days when stored in RS-I solution at 8-10 °C (1). The results of experiments using the Res-Del® technology seriously question the validity of previous pharmacological bioassay investigations conducted in *vertical* organ bath systems and/or using phosphate buffered solutions, which have been reported over the last 40 years to inhibit: glycolysis (1,2), oxidative phosphorylation, creatine kinase (3) and the enzymes involved in oxygen *free radical* scavenging (4).

(1) Rees D (1989) March: Biomesstechnik-Verlag March GmbGH 5. pp 85-94, pp 123-132.
(2) Berman DA & Saunders PR (1955) Circ Res III: 559-563
(3) Hall N & DeLuca M (1986) Adv Exp Med Biol 194: 71-82.
(4) De Frietas JM & Valentine S (1984) Biochem 23: 2079

EFFECT OF *RES-DEL®* RS-C SOLUTION ON THE VIABILITY OF ISOLATED RAT HEART PREPARATIONS OVER 1-6 HOURS OF CARDIOPLEGIC ARREST

D Rees and LJ Clissold, School of Biological Sciences, Victoria University of Wellington.

Numerous cardioplegic solutions to date have been utilised by cardiothoracic surgeons to cause cessation of both mechanical and electrical activity of the human heart during coronary by-pass surgery. The surgical procedures last for 45-100 min during which time there can be no escape from cardioplegic arrest and no ventricular fibrillation upon re-animation of the arrested heart. The criteria defined above for an *ideal* cardioplegic solution have been met using Res Del® RS-C solution containing 50 mg L^{-1} chloromycetin in preliminary experiments on the isolated *Langendorff* rat heart preparation maintained in a Res-Del® Perfusion Bath system. Initial experiments have indicated, in comparison to phosphate buffered St Thomas (STS) and Celsior® cardioplegic solutions, that recovery of RS-C cardioplegic rat hearts was far superior, giving 100-165% recovery of cardiac function following 1-6 h of cardioplegic arrest at either 20-25 °C or 35 °C with no occurrence of ventricular fibrillation. Preliminary experiments using the *non-paced, working* rat heart in a comparison of the cardiac performance during perfusion with Krebs-Henseleit (+2 mmol L^{-1} pyruvate) and RS-I solutions have indicated (1) that in RS-I mammalian solution cardiac performance was 700% greater and coronary flow rates 300% greater than those observed in Krebs-Henseleit solution.

(1) Rees D (1989) March: Biomesstechnik-Verlag March GmbGH 5. pp 85-94, pp 123-132.

REES, D. (1989):
Consideration of the inorganic and organic composition of mammalian perfusion solutions. In *Isolated Perfused Organ Preparations*. Eds. H.J. Döring, H.Dehnert. Biomesstechnik-Verlag March GmbH, Vol. **5**, pp 85-94.

IV. CONSIDERATION OF THE INORGANIC AND ORGANIC COMPOSITION OF MAMMALIAN PERFUSION SOLUTIONS

D.Rees

Historically, the design of physiological salines dates back to the thesis proposed by the great French physiologist, Claude BERNARD (1872), who, through his clinical studies put forward his treatise on the 'milieu interieur', basically purporting that to maintain the whole (person) one should ensure that the surrounding (extra)cellular environment should be balanced in all respects.

Unfortunately, the misinterpretation or misconception of Bernard's 'milieu interieur', as reviewed by ROBIN (1977), has led succeeding researchers to confuse the'extracellular' with the 'intracellular' phases of cell function and the need to maintain the cell as a 'whole'entity. The initial basic 'salt' solutions for in vitro studies commenced with the simple formulation proposed by Sidney RINGER in 1883 for the isolated perfused frog heart, after which time, similarly contrived 'salt' solutions were utilised by LOCKE (1901), TYRODE (1910) and HANKS (1948) for isolated mammalian preparations. The phosphate/bicarbonate saline originally designed by KREBS and HENSELEIT (1932) was for studies on isolated homogenates of mitochondria (organellels) from pigeon liver (KREBS and EGGLESTON, 1940) and later for his classical analysis of oxygen comsumption in tissue slices of different organs from a variety of animal species (KREBS, 1950). One would add that KREBS (1950) correctly interpreted Bernard's hypothesis that the whole cell had to retain a metabolic homeostasis.

Traditionally, phosphate/bicarbonate buffers have been used in conventional solutions for perfusing mammalian tissues and organs, with questionable validity, for over 50 years and are still being currently utilised by numerous researchers. Interestingly, it has been known for 35 years that inorganic phosphate ions inhibit glycolysis (BERMAN and SAUNDERS, 1955) and, more recently, the inhibition of creatine kinase (HALL and DeLUCA, 1985) and the enzymes involved in oxygen "free radical" scavenging which have been implicated in reperfusion injury in numerous organ systems (STEWART et al., 1986).

The ensuing four decades witnessed the use of a plethora of different saline recipes for insect, crustacea, amphibia, fish and mammals alike, of which, few have attempted to approach the natural composition of the extracellular (serum) aqueous phase for the maintenance of the physiological and pharmacological functions of isolated tissue/organ preparations.

Conceptually, it seemed logical that a basic requirement in the design of a mammalian perfusion solution with 'universal' or 'inter-species' application should, as indicated by BURTON (1975), ensure,

(a) adherence to the physiological (serum) levels of ionic and metabolic components;

(b) optimal utilisation of both residual (tissue) and added substrate, and,

(c) accurate control of temperature, gaseous exchange, pH, osmolality, conductivity and 'flow' dynamics of the perfusing solution.

Such criteria have been adopted in the formulation of RS-I mammalian solution (REES (1985)), a non-phosphate buffered medium (Fig. 1), and the utilisation of the controlled temperature and perfusion/ perifusion characteristics of the 'Res-Del Perfusion System' and the laminar flow dynamics of the Res-Del(TM) perfusion bath (Fig. 2).

In essence, a perfusion solution should attempt to maintain the myriad of continually varying electrochemical, biochemical and biophysical processes known to contribute to the overall balance of interdependancy that exists between cell metabolism and function. Our results over the last ten years

RS-I MAMMALIAN SOLUTION

(after Rees,1985)

	Component	RS-I (mOsmoles)
(IONIC)	KCl	5.0
	NaCl	110.0
	$CaCl_2$	1.2
	$MgCl_2$	0.45
(BUFFER)*	$NaHCO_3$	25.0
	BES	5.0
(SUBSTRATES)	D-glucose	10.00
	glycerol	0.11
	L-aspartate(Na^+)	0.02
	L-glutamate(Na^+)	0.30
	L-glutamine	0.40
	DL-carnitine	0.05
	choline chloride	0.01
	cocarboxylase (TPP)	0.043
	Insulin (Porcine)	25.0 mIU/L

[* aerate with 95% O_2/ 5% CO_2]

[pH 7.23 - 7.41 + 0.05 @ 20-37 °C]

Fig 1 Composition of the Res-Del™ RS-I mammalian physiological Solution

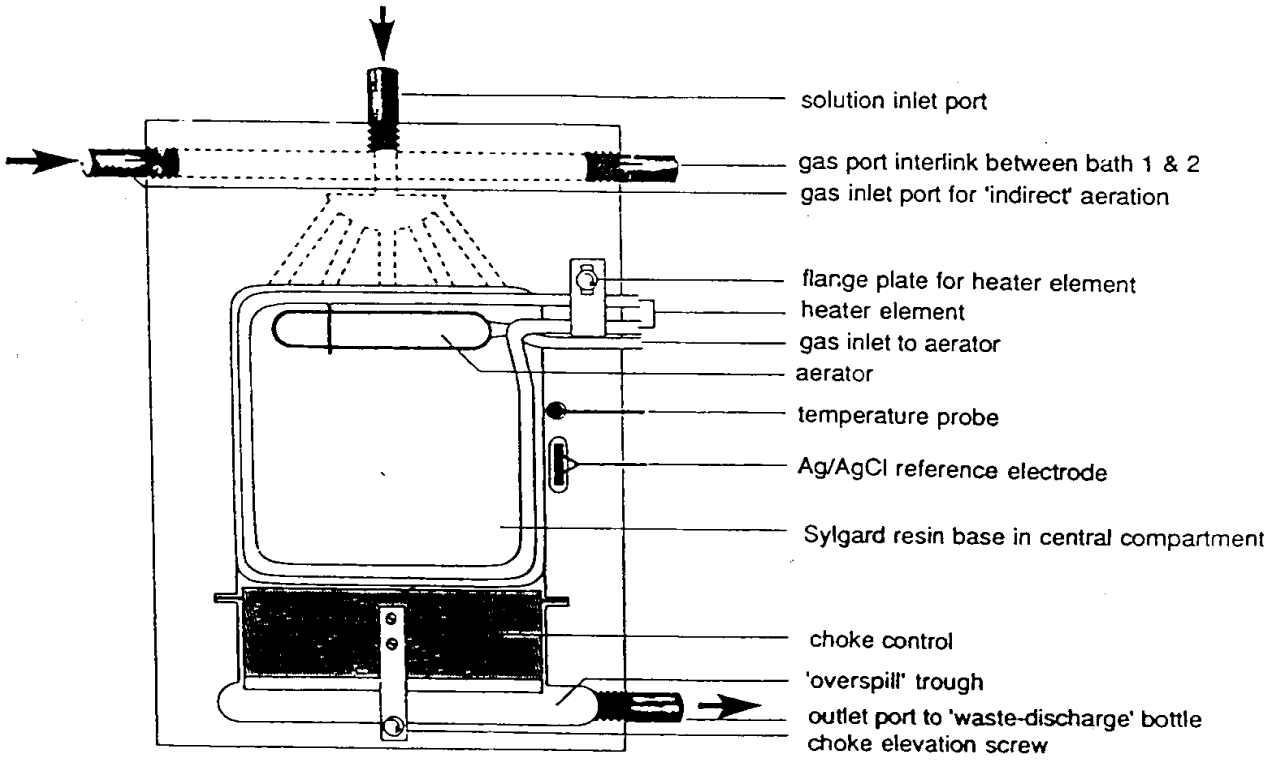


Fig 2 Res-Del perfusion bath with component modules in place

COMPONENT	SERUM	ISF	RSI (1985)	K & H (1932)
Cations (mOsmoles)				
Sodium	142	145	136	143
Potassium	4	4	5.0	5.9
Calcium (total)	2.5	2.5	1.2 *	2.5
Magnesium (total)	1.0	1.0	0.45 *	1.2
TOTAL	149.5	152.5	142.7	152.6
Anions (mOsmoles)				
Chloride	101	114	118	127
Bicarbonate	27	31	25	24.8
Phosphate (inorg.)	1.0	1.0	0	1.2
Sulphate	0.5	0.5	0	1.2
Organic acids	6.0	7.0	5(BES)	0
Proteins	2.0	1.0	0.002	0
TOTAL	137.5	154.5	148	154.2
GLUCOSE	5	-	10	11.1
INSULIN (nmoles)	1.4	-	1.7	0
OSMOLALITY (mOsmoles)	282	289?	286	299?
CONDUCTIVITY (mS cm^{-1})	12.4	12.8?	12.6	-

(* 'Freely ionised')

(? Theoretical est.)

Fig. 3 Comparison of the inorganic, organic and biophysical components of human sera and ISF with RS-I and K & H physiological solutions.

have substantiated the premise that maintaining a metabolic balance, particularly with respect to glycolysis, oxidative phosphorylation and NAD^+/NADH ratios of the cellular components of tissue/organ systems, is requisite for the validation of experiments conducted in vitro (Fig.6a & b).

Of primary importance in the design of a 'physiological' solution is to achieve a replication of the ionic profile of the extracellular phase which, in mammalian species, is identifiable with the serum and interstitial fluid (see Fig. 3). However, a common misconception has been to adopt the 'total' ionic content of the serum which does not acknowledge the activity coefficients of each ionic species (see BURTON, 1975) and, more importantly, the serum-binding of Ca^{2+} and Mg^{2+} which will ultimately determine the 'free' or 'active' ionic profile of the physiological solution (PEDERSEN, 1973). The latter condition applies in the case of RS-I mammalian perfusion solution where a conductivity value of 12.6 mS cm^{-1} compares with that of human serum (see Fig. 3). In addition, the state of hydration of the ionic components and extracellular pH (pHo) will determine the state of ionisation of the viscous interstitial phase and surface membrane constituents and is therefore a determinant factor of the molecular activity of the bioelectric membrane and the very survival of the cell per se (Fig. 4).

A basic acknowledgement in the design of RS-I mammalain solution was the fact that the cell membrane lies in continuity with a 99% gel interstitial phase (ISP) so providing a 'natural' colloidal buffer phase to excess Donnan equilibrium ionic exchange across the cell membrane (Fig. 5). In this regard, it should be noted that the osmotic pressure of human plasma is 5850 mmHg (ca. 300mOsmoles), of which, only 28 mmHg is attributable to the colloidal osmotic pressure of plasma proteins (i.e., 1.4 mOsmoles).

It may be argued therefore, that since RS-I solution is of comparable osmolality to human serum, namely, 287 mOsmoles, the additional requirement of 'plasma-expanders', such as BSA, dextran,

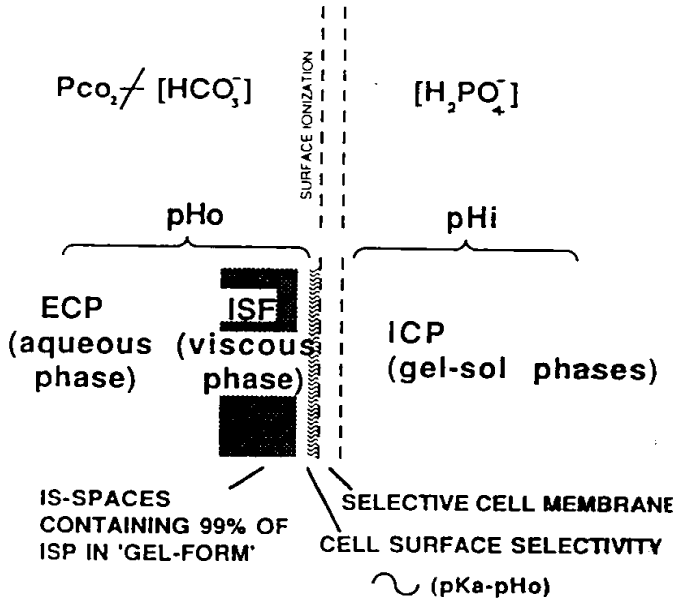
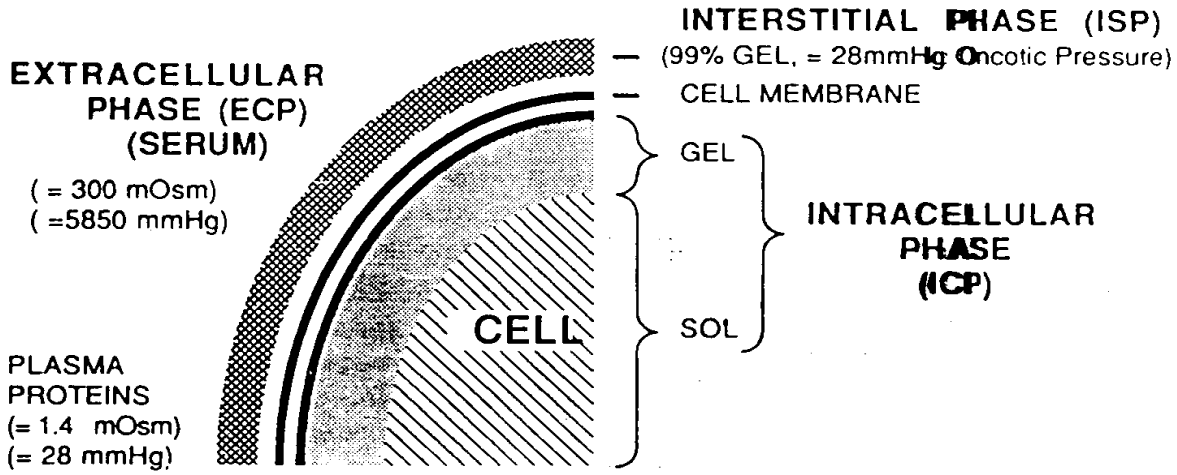


Fig. 4 Biological buffer system.



GEL = POLYELECTROLYTE 'STRUCTURAL' PHASE.
SOL = 'DYNAMIC' PHASE. METABOLIC ACTIVITY

Fig. 5 Cell interface osmotic relationships.

'Haemaccel', PVP, etc., as in other perfusion solutions, may be questioned if one considers the positive long-term results achieved utilising RS-I perfusion solution with a variety of different, isolated mammalian preparations (see Fig. 6a and b). Perhaps an exception to this theory is the mammalian kidney for our preliminary results with the perfused rat kidney have indicated that optimal glomerular filtration may necessitate the addition of crystaloid colloids to 150% of the osmotic pressure of RS-I solution for experiments lasting 4 - 24 hours to prevent oedema of the kidney tubules (BULLIVANT (1978)).

Species	Tissue/Organ	Max Days in vitro	Preparations Stored °C	Used °C
rat	jejunum	9.0	4	35
"	"	1.5	-	35
"	intestine	8.0	4	35
"	"	1.3	-	35
"	colon	5.0	-	20-35
"	uterus	3.0	-	20-35
"	"	10.0	4	35
"	detrusor muscle	2.0	-	20-35
"	diaphragm muscle	0.6	-	35-37
"	" "	2.0	-	20-35
"	soleus muscle	1.1	-	20-35
"	heart	0.3	-	35-37
"	"	1.2	-	20-35
"	heart-lung	0.7	-	35
"	RBC's	4.0	4	-

Fig. 6 a Functional viability of RS-I maintained mammalian tissue/organ preparations.

Species	Tissue/Organ	Max Days in vitro	Preparations Stored °C	Used °C
rabbit	intestine	5.0	4	37
"	"	2.0	-	20-37
"	uterus	7.0	4	37
"	superior cervical ganglion	2.0	4	37
"	" " "	0.8	-	37
"	RBC's	3.0	No haemolysis at 4°C	
guinea pig	ileum	7.0	4	37
" "	detrusor	4.0	4	37
" "	"	1.0	-	20-37
mouse	diaphragm	1.5	-	20-35
"	intestinal] n.t. release	0.9	-	20-35
"	diaphragm]	1.5	-	20-35
"	soleus	0.9	-	20-35

Fig. 6 b Functional viability of RS-I maintained mamalian tissue/organ preparations.

Nevertheless, caution is advised when contemplating the addition of such oncotic agents because of various contradictory observations reported using such plasmaexpanders, eg., BSA - antigenicity, pH and Ca^{2+} -binding ; Dextran - loss of enzyme content; 'Haemaccel' - high (6.3mM) Ca^{2+}.

Time does not permit an extensive treatment of the intricate role played by ionic species in the myriad of functions in different cell types except to remark that one should be aware of the fuctional relationships that are known to exist between the ions Na/K (membrane potential); Ca/$(Na)^2$, Ca/Mg (myofibre contractility); Ca/K, Ca/Na (voltage channels); Mg^{2+} as a cofactor with glycolytic enzymes and Ca2+ on the rate of receptor desensitization.

Essentially then, any redistribution of the Donnan equilibria of ionic constituents will result in the long-term instability of bioelectric phenomena and thereby metabolic homeostasis penultimately leading to an upset of the hydric status of any cell type and ultimately oedema and death of the isolated tissue/organ preparation.

Ultimately, ensuring that the designed physiological saline is both isotonic and isosmotic with mammalian serum has been shown in our studies to significantly increase the longevity, ie., days vs hours, and to sustain the physiological/pharmacological viability of a variety of tissues/organs from different mammalian species as shown in Fig. 6(a)(b) and Fig.10(a)(b).

Particular emphasis in the design of RS-I mammalian solution has been placed on the role of intermediate metabolism in maintaining homeostasis of cellular function.

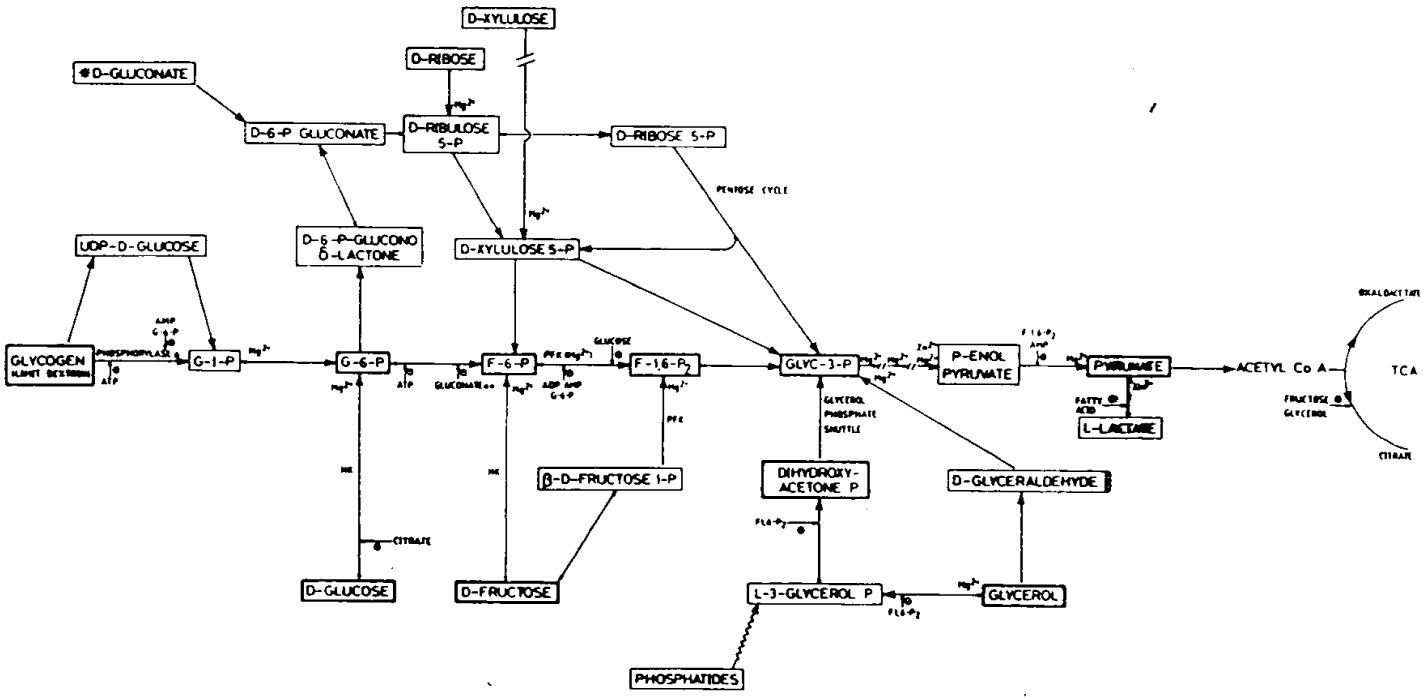


Fig. 7 Diagrammatic representation of basic glycolytic pathway in mammalian cells.

It has emerged from our results (see Fig. 6a and b) that simple maintenance of glycolysis (Fig. 7) to generate the all important acetyl Co.A and thereby a balance of NAD+/NADH, has resulted in the long-term viability of the physiological and pharmacological responsiveness in a variety of different isolated tissue/organ systems from different mammalian species (REES (1988)).

Interestingly, our studies have confirmed earlier studies namely, that the subtlety in sustaining glycolysis precludes the use of inorganic phosphate-buffered solutions because of the indications that phosphate ions interfere with the co-operativity of Mg^{2+} with the rate limiting enzymes, hexokinase and phosphofructokinase, leading to their time-dependant inactivation (cf. Fig. 10a and b). In this

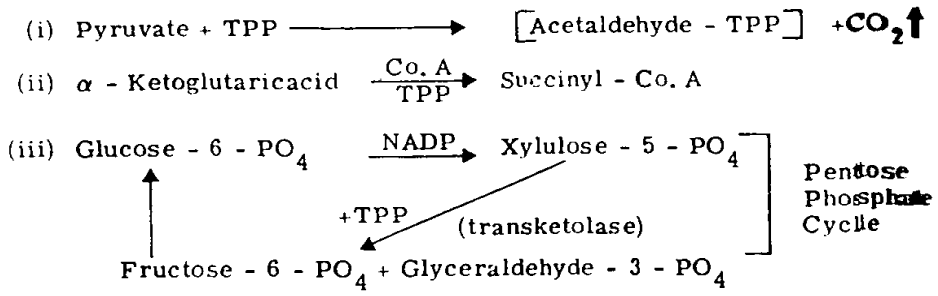


Fig. 8 The involvement of Thiamine Cocarboxylase (TTP) in the Pentose Phosphate shuttle.

context, it has been found that the inclusion of thiamine pyrophosphate, glutamate, aspartate, insulin and glycerol in RS-I solution has been beneficial in sustaining,

(a) the pentose phosphate shuttle (Fig. 8),

(b) glycerol phosphate and aspartate-malate shuttles (Fig.9), thereby optimising mitochondrial metabolism and the generation of 'high-energy' radicles.

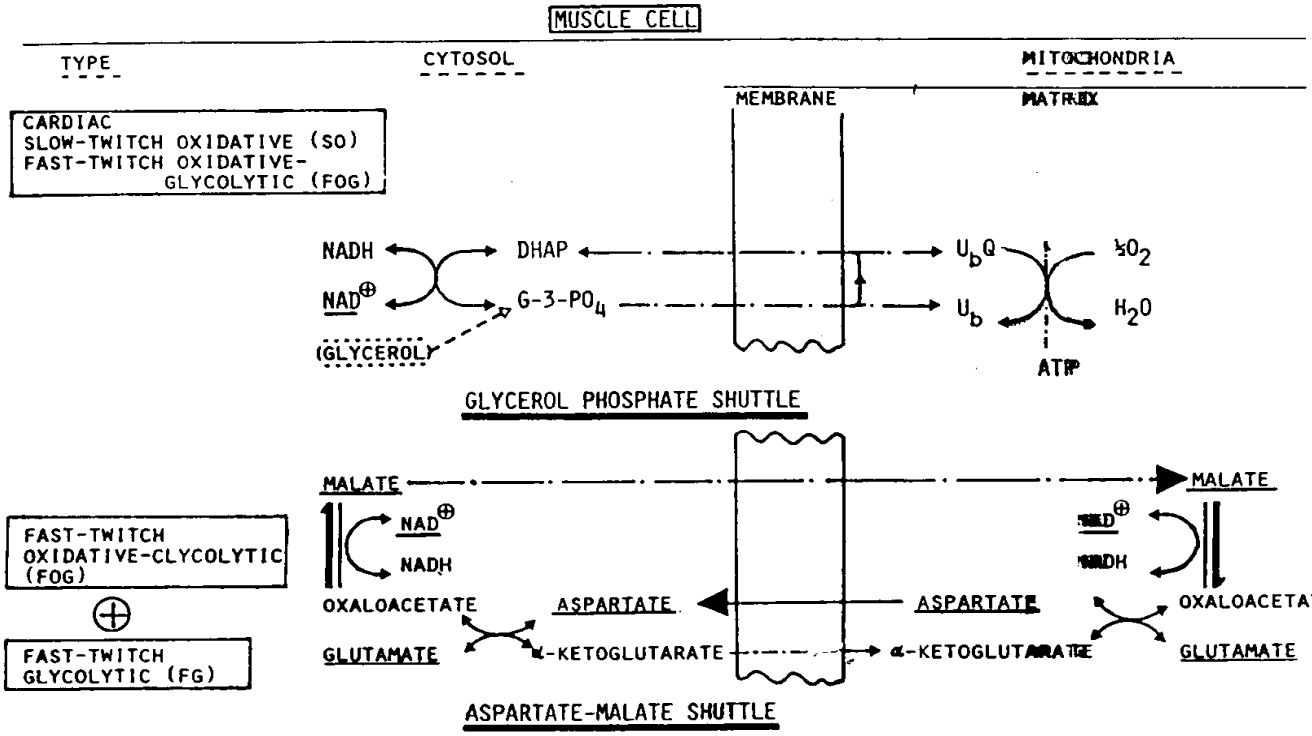


Fig. 9 NAD⁺-NADH operative shuttles in SO- FOG- & FG-type myofibres.

One of the many long-term in vitro studies conducted (see Fig. 6a and b) has involved the classical bioassay preparation, the guinea pig ileum, which has been demonstrated to retain its physiological and pharmacological characteristics for up to 7 days (see Fig. 10a and b).

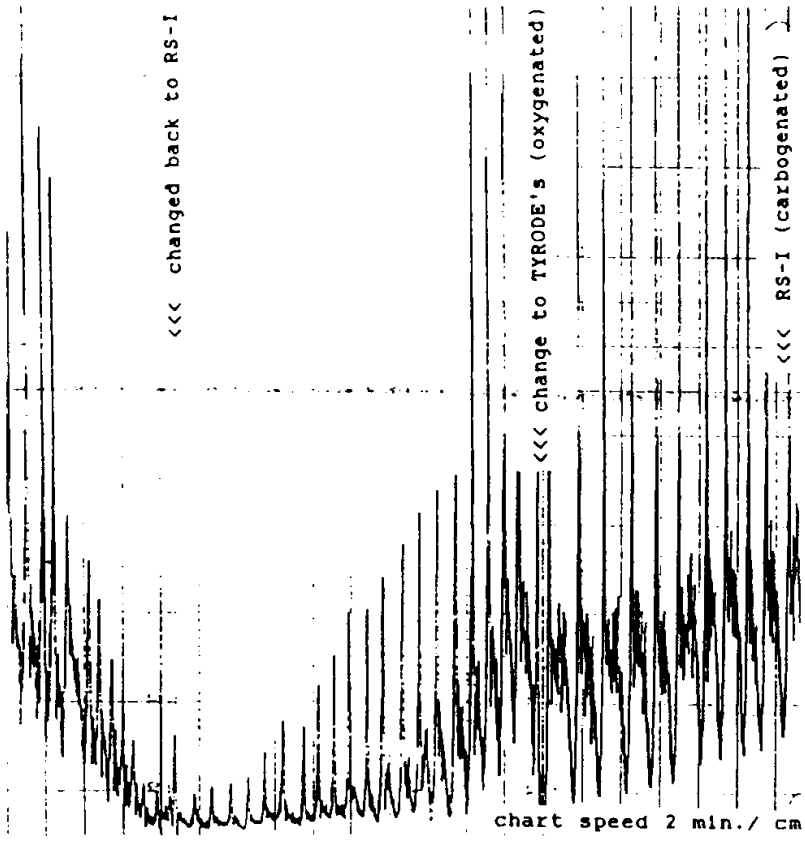


Fig. 10a **Effect of alternate perifusions of TYRODE and RS-I oxygenated salines at 37°C on electrically induced contractions of isolated, guinea-pig longitudinal ileal muscle**. Note the 90% decrease in activity over a 15 min. perifusion period in TYRODE's saline and the 100% recovery following 9 min. perifusion with RS-I mammalian saline.

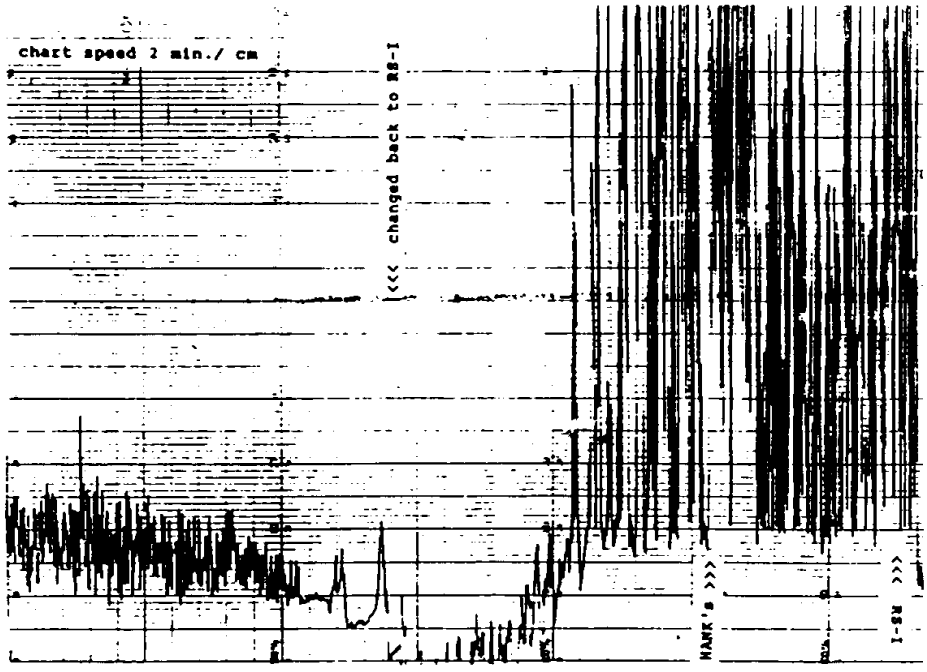


Fig. 10b **Effect of alternate perifusions of HANKS and RS-I oxygenated saline at 37°C on electrically induced contractions of isolated, guinea-pig longitudinal ileal muscle** Note the immediate decrease in contractile activity following perifusion with HANK's saline and its irreversibility after 15 min. perifusion with RS-I saline.

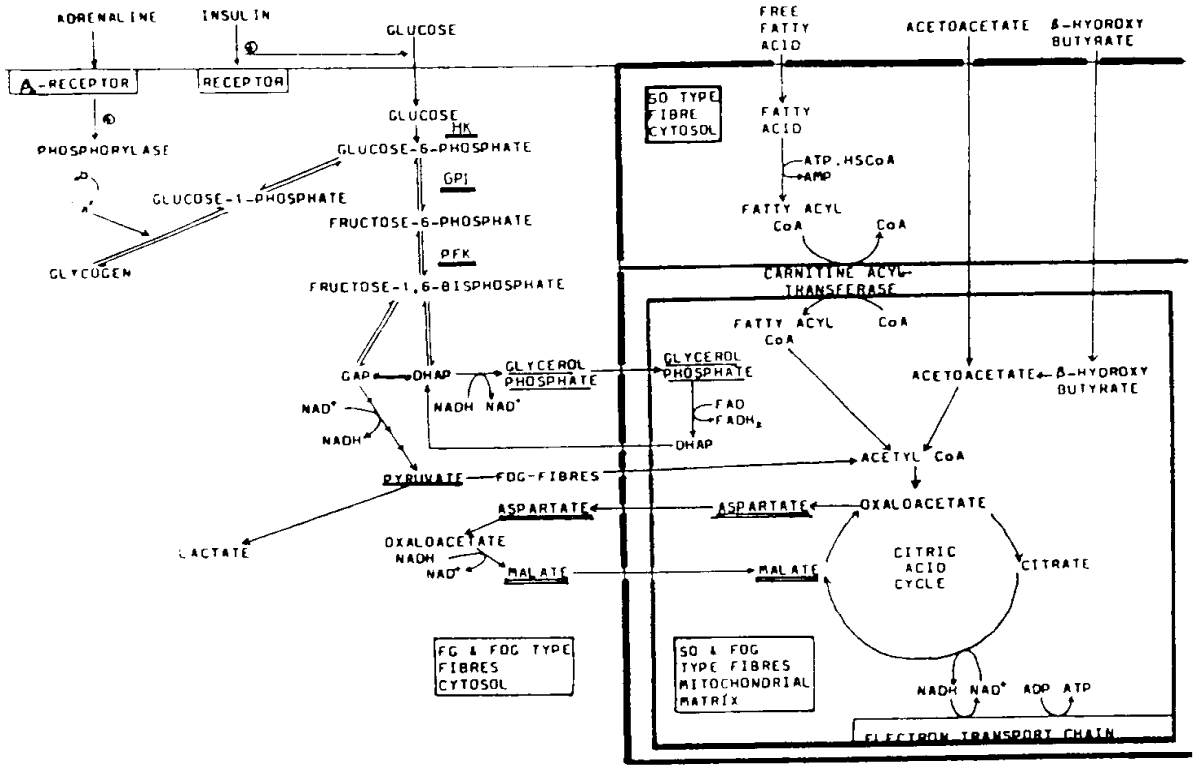


Fig 11 Schematic diagram of the salient biochemical profiles evident in SO-, FOG- and FG-type muscle fibres

Additionally, the inclusion of carnitine has proved essential in maintaining muscular contractility apparently by its ability to maximise residual fatty acid radical utilisation by the mitochondria in the form of acylcarnitine CoA and the generation of adenosine nucleotides.

We have found in our in vitro long-term studies that bacterial infection of isolated preparations occurs over periods in excess of 24 hours (Fig. 12) but has been adequately controlled by the inclusion of chloromycetin (10-50 mg/l) in RS-I mammalian solution for periods of up to 48 hours. Interestingly, electron microscopic examination of hypothermically maintained isolated rat heart preparations has indicated that occlusion of the endothelial coronary micro-vasculature was caused by bacterial deposits and not, as previously reported, red blood cell sludging (M.J.TOES, unpublished data). Preliminary studies have indicated that negligible clumping of red blood cells occurs in RS-I mammalian solution for periods of up to 48 hours (REES, unpublished data).

In conclusion, it is suggested that it is the maintenance over time of the cell isovolume that must be preserved as this directly relates to metabolic homeostasis and therein the preservation of physiological and pharmacological viability of in vitro preparations.

SUBSTRATE	TIME (hr)	BACTERIA CULTURED: Pseudomonas aetuginosa	Flavobacterium spps.	Enterobacter clocae	Klebsiella edwardsii
De-ionised H_2O	0.0	+	-	-	-
RS-saline	1.0	+ +	+ +	-	-
RS-saline	8.0	+++	+ +	+ +	-
RS-saline	20.0	+ +	-	-	+ +

+ ; light growth
++ ; moderate growth
+++; heavy growth
- ; no growth

Fig 12 Time-Dependant bacteriological profile of physiological perfusion medium

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II. PERFUSED RAT KIDNEY PREPARATION

D. Rees

I. Introduction

Before describing the RES-DEL equipment and experimental protocol adopted for the perfusion/perifusion of RS-I mammalian solution (Fig. 1) using the isolated rat kidney, I should like to briefly describe the basic components of the Res-Del Perfusion system and the versatility of the horizontally designed Res-Del bath in its ability to accomodate a variety of different perfused/perifused isolated tissue/organ preparations.

RS-I MAMMALIAN SOLUTION

(after Rees,1985)

	Component	RS-I (mOsmoles)
(IONIC)	KCl	5.0
	NaCl	110.0
	$CaCl_2$	1.2
	$MgCl_2$	0.45
(BUFFER)*	$NaHCO_3$	25.0
	BES	5.0
(SUBSTRATES)	D-glucose	10.00
	glycerol	0.11
	L-aspartate(Na^+)	0.02
	L-glutamate(Na^+)	0.30
	L-glutamine	0.40
	DL-carnitine	0.05
	choline chloride	0.01
	cocarboxylase (TPP)	0.043
	Insulin (Porcine)	25.0 mIU/L

(* aerate with 95% O_2/ 5% CO_2)

(pH 7.23 - 7.41 + 0.05 @ 20-37 °C)

Same as C.IV

Fig. 1: Composition of Res-Del RS-I-solution

The Res-Del bath affords a means of simultaneous perfusion and perifusion of isolated organs or simple perifusion of tissue biopsies from the mouse, rat, guinea pig or rabbit for physiological and pharmacological functional assessment.

Basically, the design of this horizontal bath has been shown to maintain isolated organ/tissue preparations at temperatures of 10-37°C by direct heating of the saline in the inner bath compartment with the temperature monitored, and thereafter controlled, by a microprocessor designed temperature unit. Direct aeration of the perfusing saline is maximised by a glass sintered aerator module and provides optimal mixing of the saline ingredients or 'test' substances.

The translucent, open design of the RES-DEL bath in combination with the optically transparent Sylgard resin floor makes fixation of isolated preparations and on-going microscopic examination, surgical, physiological and pharmacological manipulations an easy task for technicians, students and research professionals. The patented slit-flow design and weir-choke spillway facilitates a unique non-vibrational, laminar-flow exchange of saline for even the most delicate of preparations even

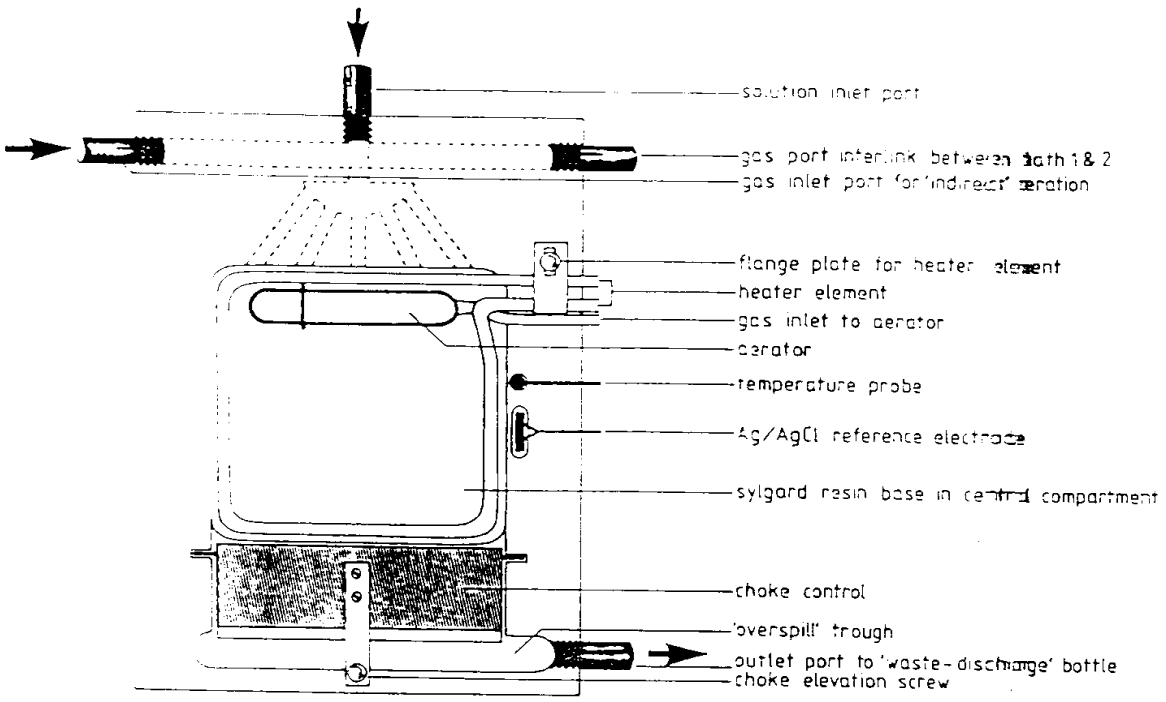


Fig. 2 RES-DEL Bath

during rapid perifusion rates of 4-1000 cm^3/min. The latter facility has proved most benificial during perifused or microphoretic applications of pharmacological agents.

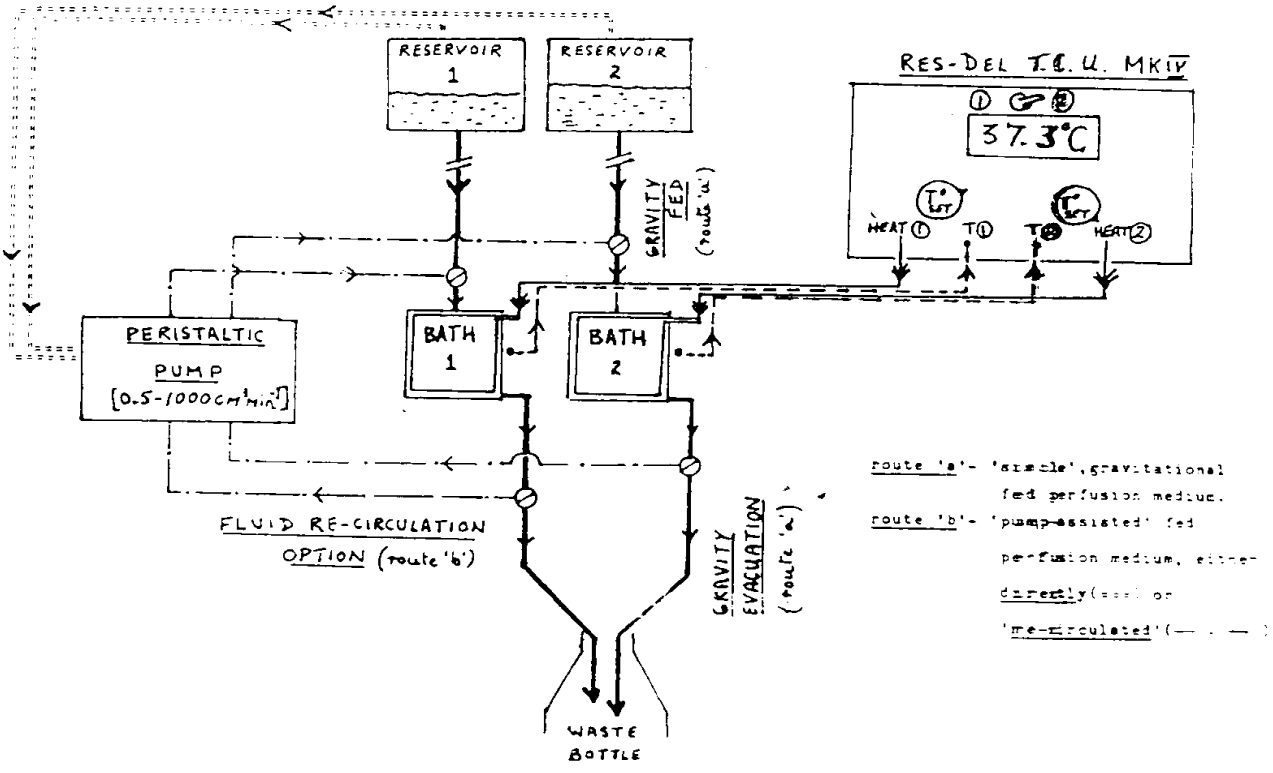


Fig 3 a RES-DEL Perfusion Set-up

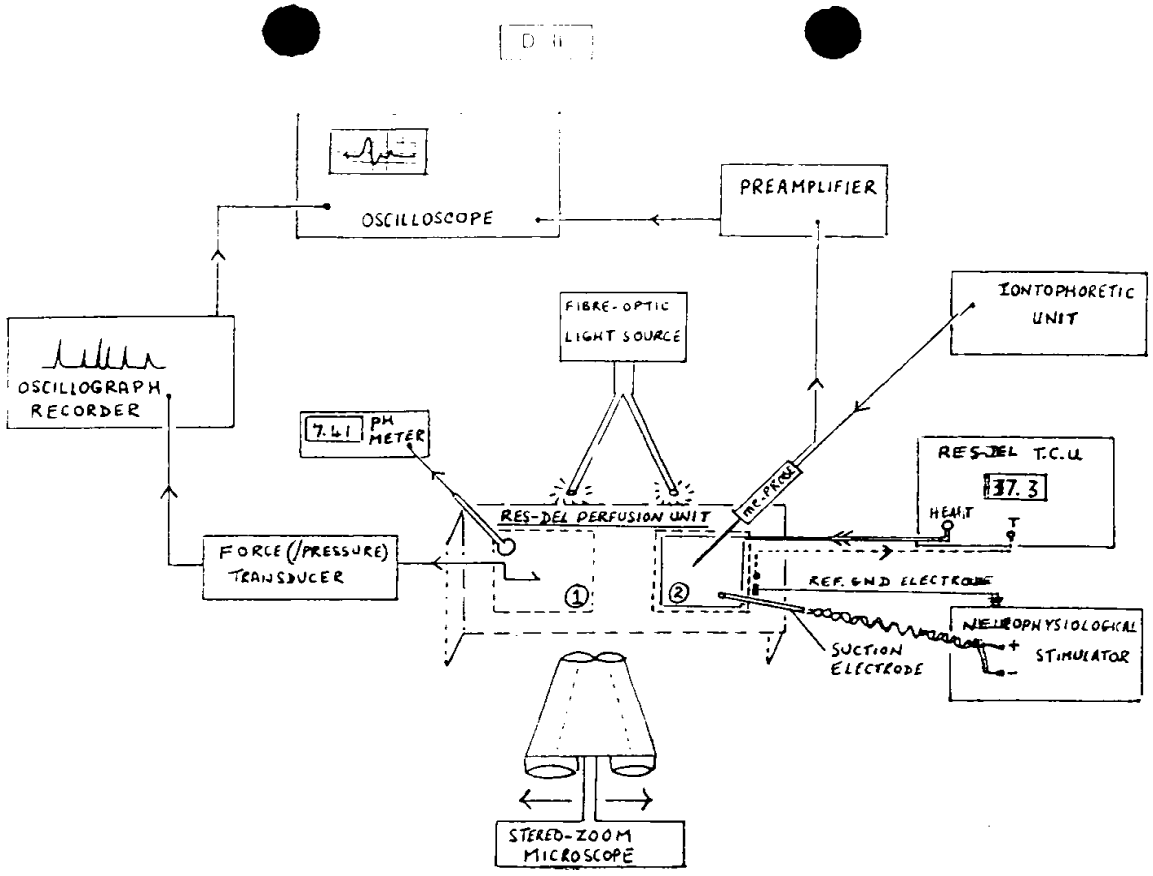


Fig. 3 b: Placement of RES-DEL Perfusion Set-up

The perfusion system has been designed to allow simple gravitational or pump-assisted fluid input and more importantly, simple, gravitional fluid overflow so eradicating the need for currently used cumbersome and troublesome suction-pump techniques (Fig.3b). In addition, the system occupies a central position to, and is compatible with existing conventional apparatus so accentuating equipment currently under-utilised with conventional, vertical isolation bath systems.

Fig. 4 to 9 indicate how the basic features of the RES-DEL bath have been used for the examination of different types of isolated organs and tissues in student laboratory exercises and more demanding research studies.

II. Application of the RES-DEL Perfusion System

In Fig. 4 the classical phrenic nerve-hemidiaphragm muscle preparation is shown diagrammatically as an example of a skeletal nerve-muscle preparation per se and illustrates how simultaneous mechanical and/or electrophysiological (microelectrode) measurements may be achieved. Additionally, perifused or microphoretic (localised) application of 'test' drugs can be performed during electrical stimulation of the preparations by either indirect (neural) stimulation of nerve fibres via suction electrodes (Fig. 5) or direct field/transmural stimulation using conventional platinum electrodes.

In Fig. 6 the ability of the RES-DEL system to facilitate 'control' vs 'experimental' examination of such preparations is depicted.

The identical experimental set-up can perform comparable examination of the physiological/pharmacological characteristics of gastro-intestinal/urino-genital tract and blood vessel preparations of even the smallest of biopsies. The ability of the suction electrodes (Fig. 5) to electrically stimulate fine nerve branches in the perivascular beds of visceral preparations has proved an invaluable tool in pharmacological bioassay experiments.

In this mode of operation fixed cannulae have allowed the Res-Del bath to be utilised to study the classical 'Langendorff' and/or 'working' rat heart preparation (Fig. 8a) or even a combined heart-lung

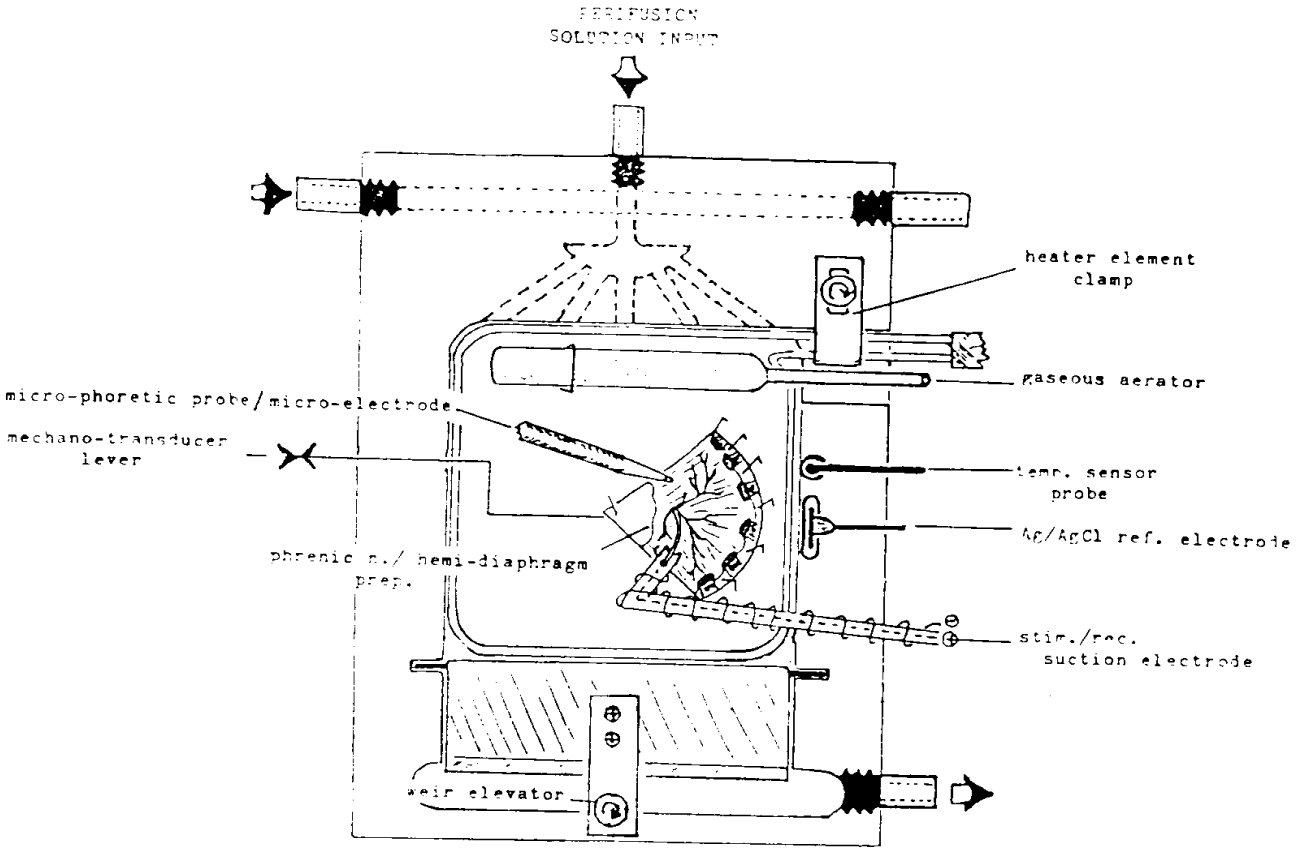


Fig 4 Skeletal of the RES-DEL perfusion system

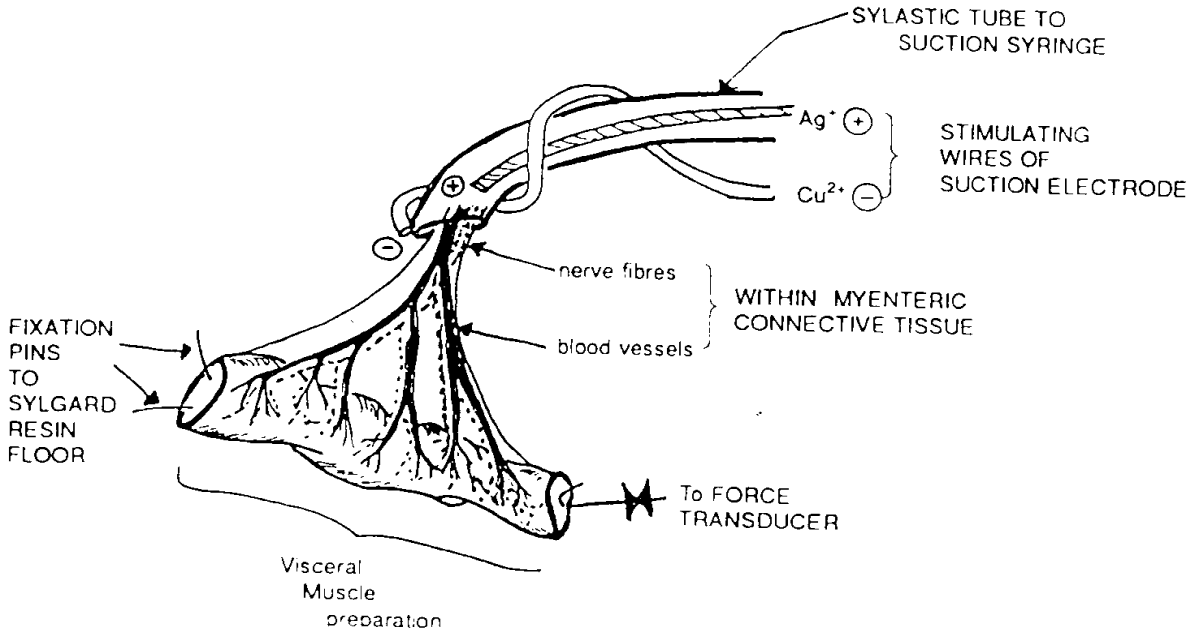


Fig 5 Suction electrode arrangement

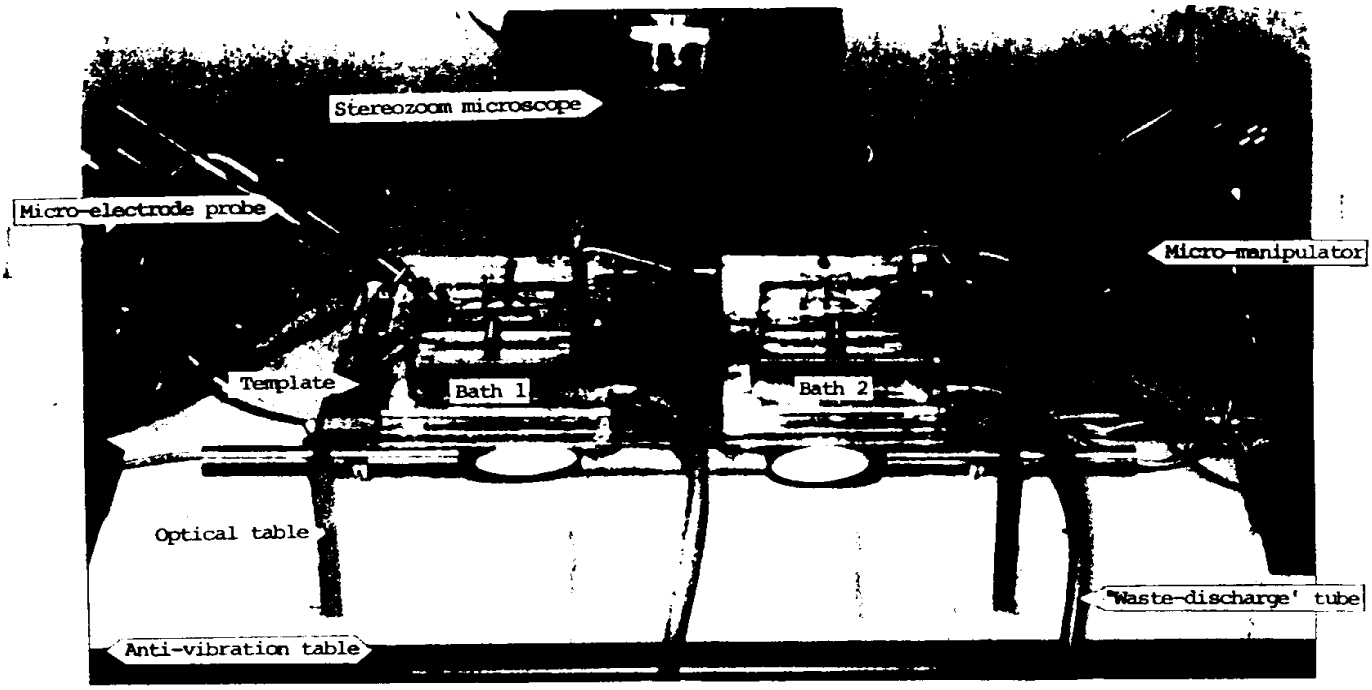


Fig. 6: RES-DEL perfusion system with conventional neurophysiological accessories

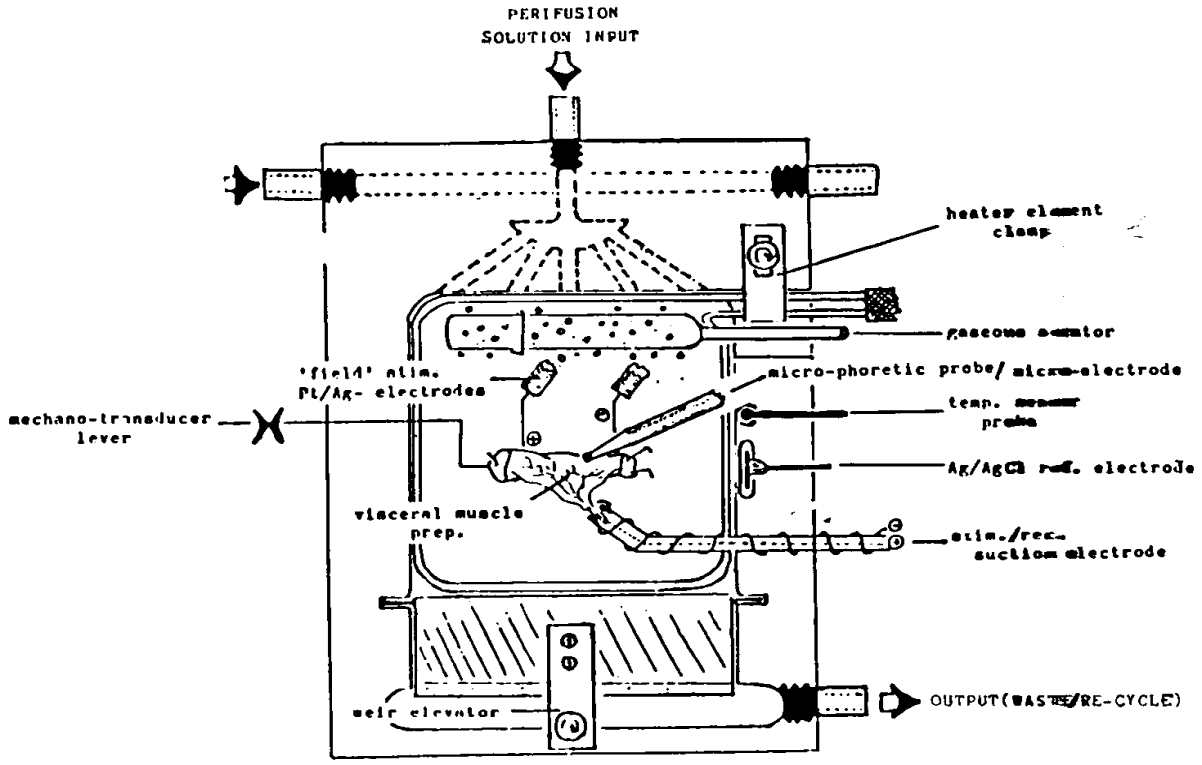


Fig. 7: Visceral muscle preparation

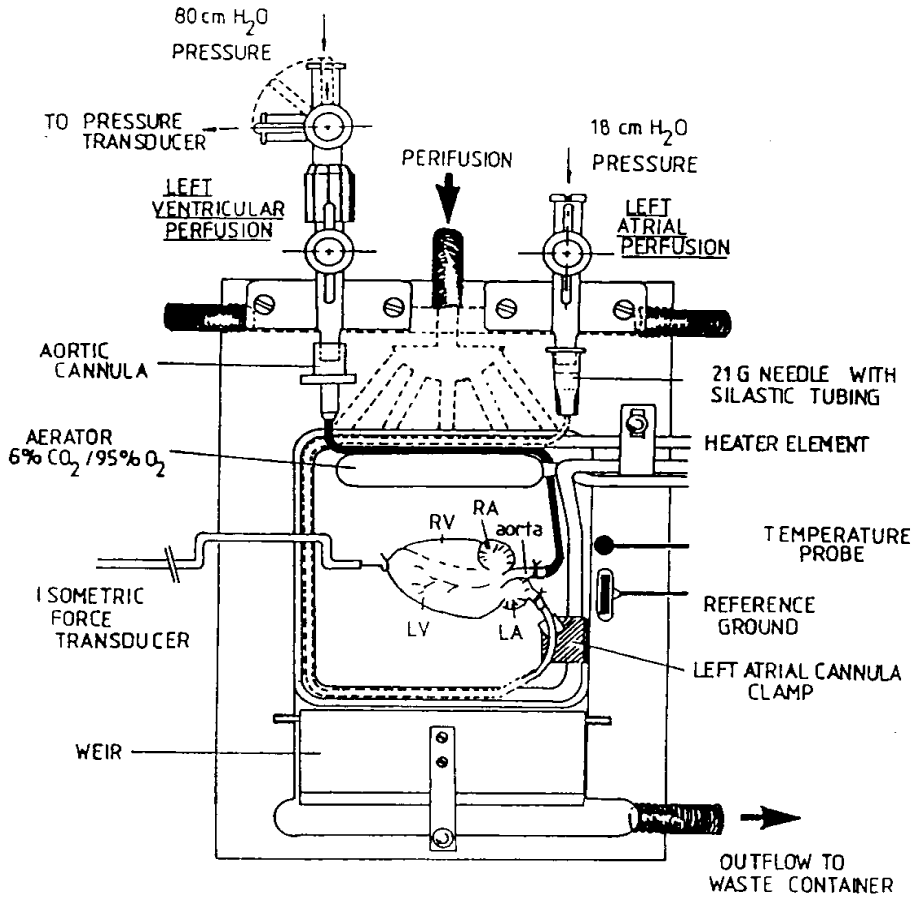


Fig. 8 a: RES-DEL isolated heart preparation

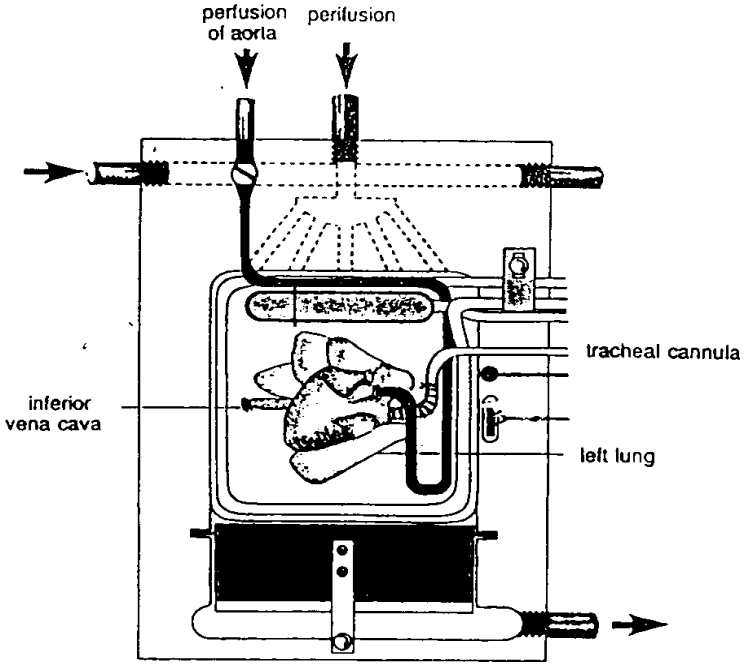


Fig. 8 b: Heart-lung preparation

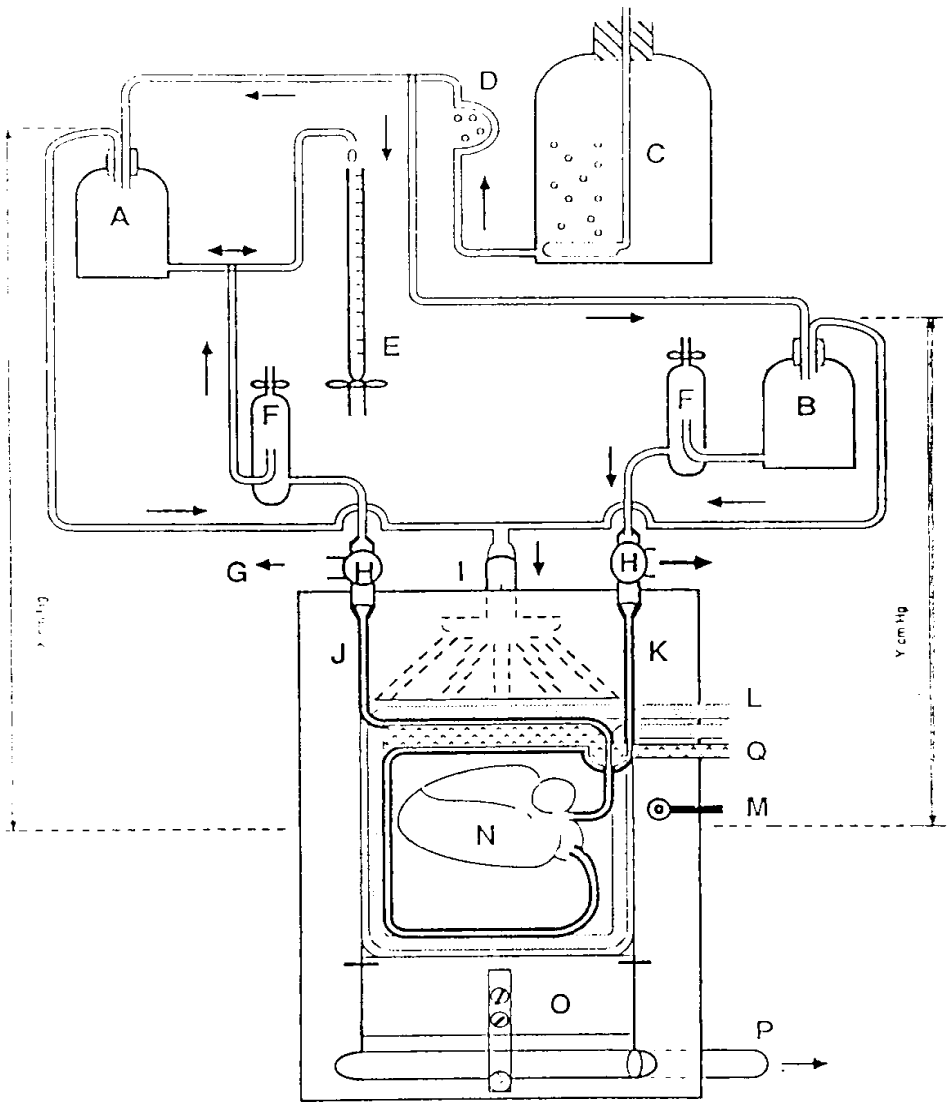


Fig. 8 c: Perfusion Setup for heart preparations

preparation (Fig. 8b) for periods of up to 48 hours when RS-I mammalian saline has been the perfusing medium.

Simple gravitional or pump-assisted feed can be used for this type of preparation as depicted diagrammatically in Fig. 8c. A video presentation demonstrating these techniques will be available during this workshop session.

In Fig. 9 a) and b) the use of suction electrodes and ECG electrodes are demonstrated for the use with such isolated heart preparations because of the "open" design of the bath and the ability, using a microscope, to position such electrodes accurately on the isolated heart. The general arrangement for this procedure is depicted in Fig. 9a) while Fig. 9b) details how other pressure and electrical measurements could be achieved.

The basic alignment and set-up of the perfused kidney preparation is comparable to that previously described for the rat heart preparation. Initially, gravitational perfusion may be used to flush residual blood from the renal vessels but this mode of perfusion of the renal artery must be replaced by

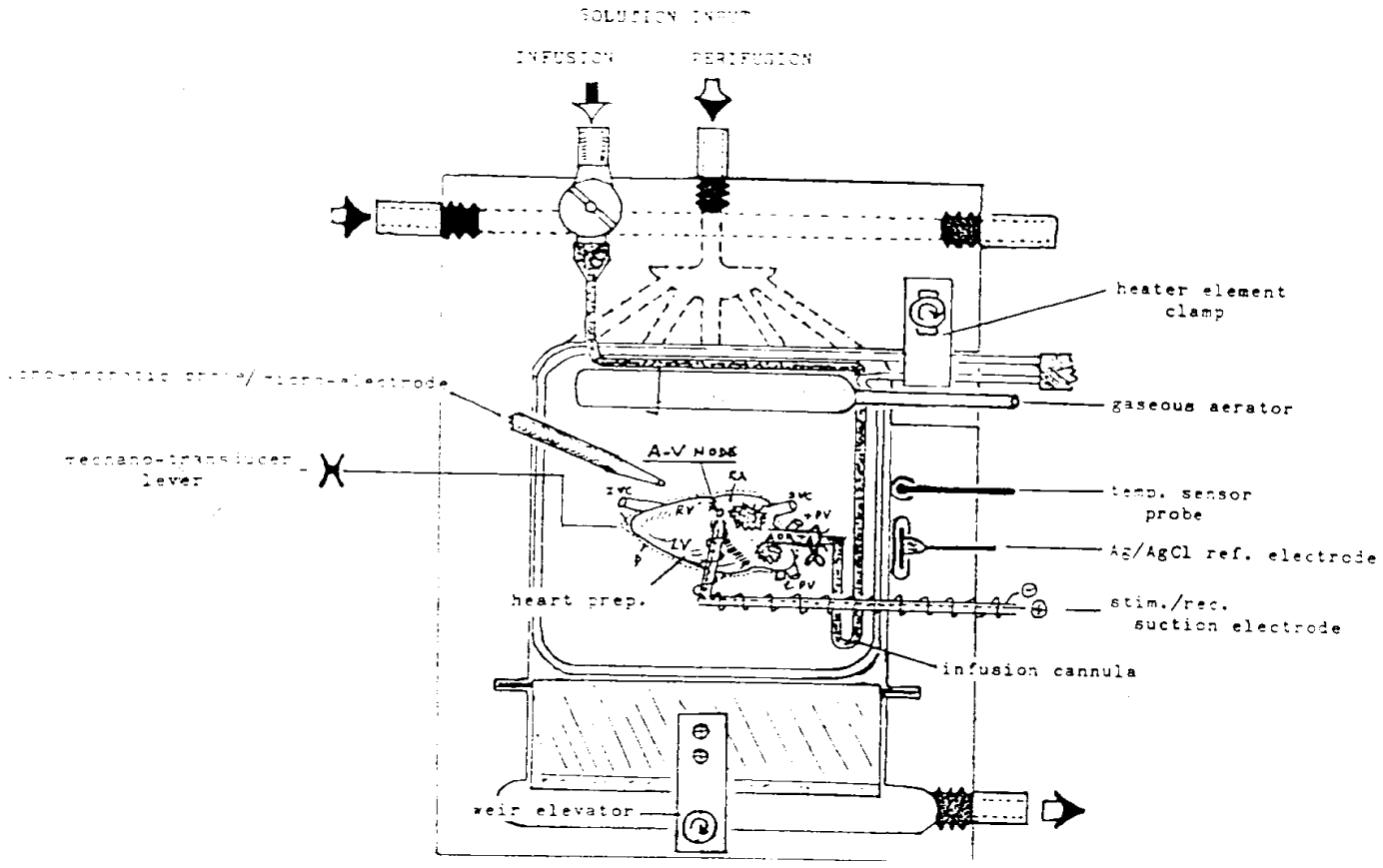


Fig 9 a Stimulation (Recording) arrangement for heart

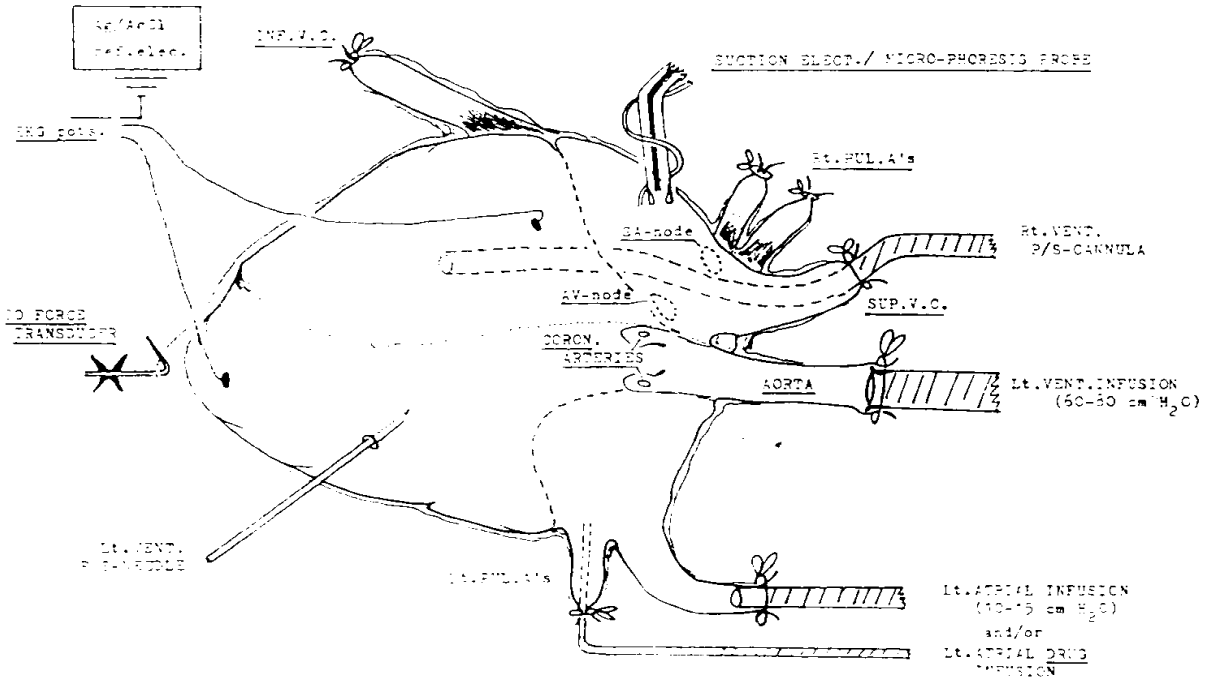


Fig 9 b Stimulation (Recording) arrangement for heart

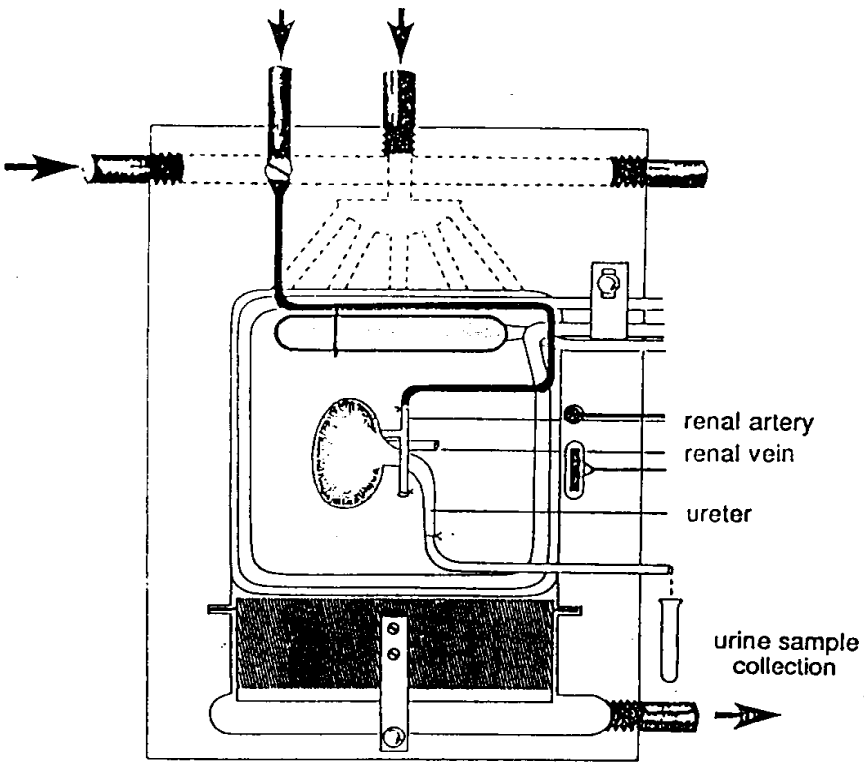


Fig. 10: Rat Kidney Preparation

pump-assisted delivery of the perfusion saline as perfusion pressures of 60 - 220 mm Hg are required to achieve optimum glomerular filtration in the isolated kidney preparation.

III. Experimental Procedures

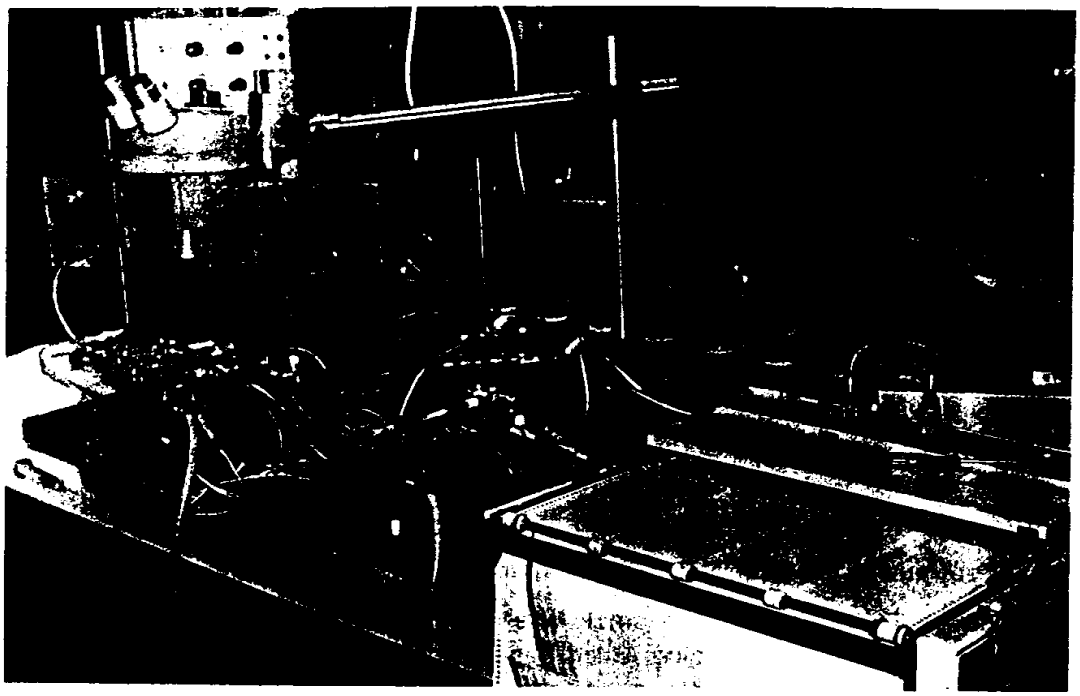
1. Anaesthetize rat with 'Sagatal'(Sodium pentobarbitone) and place in supine position.
2. Expose left URETER and make a small hole with a 27G needle half way down the ureter; cannulate ureter using PE10 tubing which has been drawn to a point and cut at 45o; secure ligature. Cut tube to a length of 10 cm and place outside the bath into a vial for the collection of urine.
3. Expose left KIDNEY, locate DESCENDING aorta and clamp.
4. Place open ligatures around RENAL artery and vein.
5. Free the renal artery and vein and transect at their respective junctions with the DESCENDING aorta and INFERIOR vena cava.
6. Remove kidney with cannulated ureter from the rat by grasping the surrounding fatty connective tissue. Secure to the Sylgard resin floor using fine steel pins through fatty connective tissue.
7. Cannulate RENAL artery using a winged 25G infusion blunted needle and ligature securely in place. Fill bath with RS-I saline and aerate.
8. Commence initial pump-assisted perfusion at 2 cm3/min.
9. Cannulate renal VEIN with PE60 tubing, ligature and secure to edge of the bath to facilitate renal outflow monitoring.

10. The PUMP- ASSISTED perfusion rate can now be adjusted to ensure optimal perfusion pressure and renal artery flow dynamics for the subsequent analyses of kidney function.

11. Using the temperature control unit select the desired experimental perfusion fluid temperature within the Res-Del bath.

* * * * * * * * * * *

Unfortunately, we are unable to provide a comprehensive analysis of rat kidney function because we have not beenprovided with the results conducted at Guys' Hospital,London, using one of our Res-Del machines. However, inpreliminary experiments we have been able to achieve urineflows of 10 - 130 µL/min. at renal inflow pressures of 60 210 mm Hg for periods of up to 18 hours over a temperature range 22–37°C.



Workshop foto for the setup for isolated organs, here demonstrated with the isolated perfused kidney.

PROCEEDINGS OF THE PHYSIOLOGICAL SOCIETY, FEBRUARY 1978, 278, 8-9P

A non-phosphate-buffered physiological saline for *in vitro* electrophysiological studies on the mammalian neuromuscular junction

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There have been numerous attempts to design an 'ideal' physiological mammalian saline (see R. F. Burton, 1975). In this study, particular emphasis has been placed on designing a balanced ionic and metabolic (substrate) saline approaching that of mammalian sera (Table 1) which would be capable of maintaining 'active' metabolism in *in vitro* nerve-muscle preparations. An inappropriate saline might accentuate impaired neuromuscular transmission of preparations from myasthenic patients or from animals with experimentally induced myasthenia gravis.

The suitability of R-S (Rees-Simpson) saline for long-term (20–50 hr) *in vitro* experiments was further facilitated by replacing sodium dihydrogen phosphate with

Table 1. Comparison of ionic and substrate components in mammalian sera and R-S saline

	Serum			
	Mouse*	Rat*	Human†	R-S saline‡
	Ions (mM)			
Na^+	152·0	144·5	143·4	135·0
K^+	6·2	6·1	4·4	5·0
Cl^-	114·5	98·0	102·7 [100–37]	121·0
Ca^{2+}	2·4		2·5	2·0
Mg^{2+}	1·3		0·94	1·0
PO_4^{2-}	2·4		1·4	—
HCO_3^-	12·0	24·0	24·9	20·0–25·0
	Substrate components			
Glucose (mmol)	10·4	5·4	4·6	11·0
Pyruvate (mmol)	0·14	0·22	0·58	—
Lactate (mmol)	4·8	4·1	1·1	—
Glutamic acid (mmol)	—	—	0·47	0·30
Glutamine (mmol)	—	—	0·44	0·40
Insulin (m-i.u.)	37·0	17·3	20·0	—
Choline (mg/l.)	—	—	4·4	1·0–5·0
Cocarboxylase (μg/l.)	—	—	40·0	20·0

* Experimental values.

† From *Documenta Geigy*, 7th ed.

‡ RS-saline buffered with 5·0 mmol BES. Oxygenated with 95% O_2 + 5% CO_2 for 40 min. pH: 7·24 (reservoir) to 7·39 (bath) at 25 °C.

a Good's buffer, BES (Good, Winget, Winter Connolly, Izawa & Singh, 1966), thereby avoiding precipitation of calcium phosphate and pH instability. This saline has now been used successfully with *in vitro* nerve-muscle preparations from mouse, rat, rabbit, dog, horse and human muscles. A detailed electrophysiological study on nerve–muscle preparations of the mouse diaphragm, intercostal, soleus and peroneous muscles indicated no deleterious changes in neurophysiological parameters over periods of 30 hr.

Supported by a Research Fellowship from the Muscular Dystrophy Group of Great Britain.

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British Journal of Anaesthesia 1991, 67: 447–451

INTERACTION BETWEEN BETAMETHASONE AND VECURONIUM

S. M. PARR, B. J. ROBINSON, D. REES AND D. C. GALLETLY

SUMMARY

A possible interaction between betamethasone and vecuronium was examined in 20 rat phrenic nerve–hemidiaphragm preparations. Ten preparations were bathed in a physiological solution with betamethasone 1 μmol litre^{-1} added and, after a 30-min period were exposed to vecuronium at concentrations of 4, 6, 8 and 10 μmol litre^{-1} with vecuronium free washings between each exposure. Ten control experiments were performed also using a betamethasone-free bathing solution. In comparison with control, the betamethasone group had significantly (P = 0.0008) less depression of muscle contraction (twitch) force at all concentrations of vecuronium. The calculated ED_{50} (50% depression of muscle contraction force) was 5.65 μmol litre^{-1} for controls and 7.39 μmol litre^{-1} for betamethasone-pretreated preparations. This study confirms our previous clinical observations that an interaction occurs between vecuronium and betamethasone which is characterized by resistance to neuromuscular block.

KEY WORDS

Interactions: betamethasone, vecuronium. Neuromuscular relaxants: vecuronium.

Steroids have been known for several years to facilitate neuromuscular function, and steroid induced resistance to competitive neuromuscular blocking drugs has been noted in three previous case reports [1–3]. Recently we reported two neurosurgical patients treated with betamethasone who had apparent resistance to vecuronium, and in a subsequent review of anaesthesia case records we found that patients treated with betamethasone required on average, 75% more vecuronium than patients not treated [4]. In this study we have attempted to demonstrate an interaction between betamethasone and vecuronium in a rat phrenic nerve–hemidiaphragm preparation.

MATERIALS AND METHODS

Male Dark Agouti rats (8–10 weeks) weighing 150–200 g were killed by cervical dislocation. The right and left hemidiaphragms with accompanying phrenic nerves were dissected out, placed horizontal in individual Res-Del organ baths and bathed with a physiological solution (RS-1 mammalian solution) aerated with 5% carbon dioxide in oxygen [5]. The composition of the solution is summarized in table I.

The bathing temperature was maintained at 34.5 ± 0.5 °C and the muscle stretched to a basal preload tension of 4 *g*. Each preparation was attached to an isometric force transducer inserted through the central tendon of the hemidiaphragm and was stimulated indirectly using suction electrodes attached to the phrenic nerve, with a continuous biphasic double pulse (12-ms pulse interval) square wave at a supramaximal voltage of 0.1 ms duration at 0.2 Hz. Short periods (30 s) of direct muscle stimulation (supramaximal monophasic stimulation at 5 Hz) were performed also using muscle electrodes to exclude any local action of betamethasone or vecuronium on the muscle. Contractions were recorded isometrically and displayed on a pen chart recorder.

S. M. PARR, B.M., F.C.ANAES.; B. J. ROBINSON, B.SC., M.SC.; D. C. GALLETLY, F.F.A.R.A.C.S., F.C.ANAES.; Section of Anaesthesia, Wellington School of Medicine, P.O. Box 7343, Wellington South, New Zealand; D. REES, B.SC., PH.D., School of Biological Sciences, Victoria University, Wellington, New Zealand.Accepted for Publication: March 21, 1991.

Correspondence to D.C.G.

TABLE I. *Solute composition and concentration of the physiological solution. BES = N,N-bis[2-hydroxyethyl]-2-aminoethanesulphonic acid*

Na^+ 136.0 mmol litre $^{-1}$	Glycerol 0.11 mmol litre $^{-1}$
Cl^- 118.0 mmol litre $^{-1}$	L-Aspartate 0.02 mmol litre $^{-1}$
K^+ 5.0 mmol litre $^{-1}$	L-Glutamate 0.30 mmol litre $^{-1}$
Ca^{2+} 1.2 mmol litre $^{-1}$	L-Glutamine 0.40 mmol litre $^{-1}$
Mg^{2+} 0.45 mmol litre $^{-1}$	DL-Carnitine 0.05 mmol litre $^{-1}$
HCO_3^- 25.0 mmol litre $^{-1}$	Choline 0.01 mmol litre $^{-1}$
BES 5.0 mmol litre $^{-1}$	Cocarboxylase (TTP) 0.043 mmol litre $^{-1}$
D-Glucose 10.0 mmol litre $^{-1}$	Insulin (porcine) 25 miu litre $^{-1}$

Where possible, the preparations were used in pairs from the same rat, in independently perfused double organ baths with equal numbers of left/right hemidiaphragms used for each group. The flow rate of the perfusate was 2 ml min^{-1}. At least 30 min was allowed for a steady state to be achieved; muscle contraction (twitch) force measurements produced by indirect and direct stimulation of preparations in the absence of steroid or neuromuscular blocker were recorded. One of the hemidiaphragm preparations was exposed to betamethasone 1 μmol litre^{-1} for the duration of the investigation. All changes of perfusate solution commenced with a 40-ml flush (the volume of the organ bath) followed by perfusate 2 ml min^{-1}. Control and betamethasone treated preparations were exposed to vecuronium at concentrations of 4, 6, 8 and 10 μmol litre^{-1}. Muscle contraction force (both direct and indirect) was recorded 30 min after each change of perfusate containing vecuronium. Vecuronium-free solutions were used to wash the preparations until twitch height returned to control values, at which time the next vecuronium concentration was added.

To ensure that preparations had not deteriorated during the experimental period, data were not included in the analysis if the response to direct stimulation had decreased at the end of the experiment.

Statistics

Data from control and betamethasone groups were compared using analysis of covariance, with the dose of vecuronium as the covariate and terms for the control or betamethasone groups and for individual preparations within these groups. A test for difference in slope between the control group and betamethasone group was made by including a dose × group term in the model.

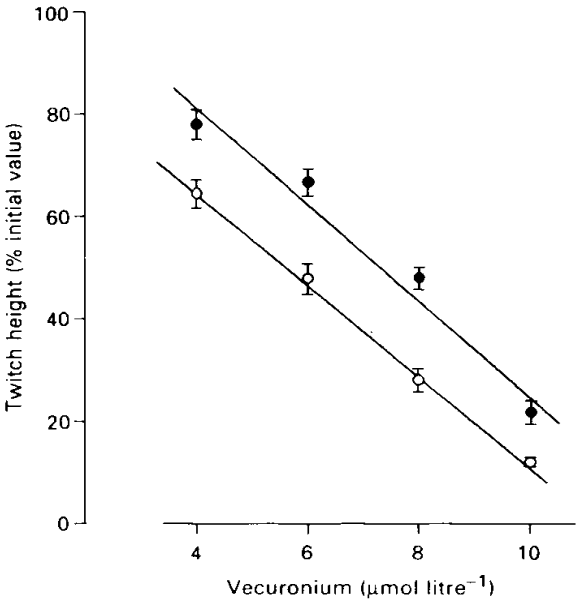

FIG. 1. Dose–response relationship for control (○) and betamethasone–pretreated (●) preparations (mean, SEM). The ED_{50} for 50 % depression of twitch height was 5.65 μmol litre^{-1} for control and 7.39 μmol litre^{-1} for betamethasone-pretreated preparations.

RESULTS

Satisfactory data were obtained from 20 hemidiaphragm preparations. Ten received betamethasone and 10 acted as control. No significant difference was observed in indirectly or directly stimulated mean twitch height before and after addition of betamethasone.

The slopes of the dose–response lines to vecuronium in control and betamethasone treated preparations were not significantly different ($P = 0.14$) (fig. 1). When a common slope was fitted, in

comparison with control, the betamethasone group showed significantly ($P = 0.0008$) less depression at all concentrations of vecuronium examined. The calculated ED_{50} for 50% depression of twitch height was 5.65 μmol litre^{-1} for controls and 7.39 μmol litre^{-1} for betamethasone pretreated preparations. Thus the addition of betamethasone increased the ED_{50} for vecuronium by approximately 30%.

DISCUSSION

In 1944, Torda and Wolff demonstrated that neuronal synthesis of acetylcholine could be increased by prior administration of adrenocorticotrophic hormone (ACTH) [6]. They demonstrated also that, in the absence of ACTH (hypophysectomized rats), there was reduced acetylcholine synthesis resulting in neuromuscular dysfunction, which was restored following administration of ACTH [7].

Recent case reports suggest that an interaction between corticosteroids and the neuromuscular junction may have clinical implications for the anaesthetist. There have been two case reports of partial recovery from pancuronium given in association with steroid therapy [1, 2] and a case of resistance to vecuronium in a patient receiving long-term testosterone [3]. Following two cases of unexpected movement during neurosurgery in patients receiving vecuronium and pretreated with betamethasone, we performed a retrospective review of 50 neurosurgical patients, examining the doses of vecuronium administered to patients with and without betamethasone pretreatment. Those data suggested that patients pretreated with betamethasone were resistant to the neuromuscular blocking properties of vecuronium and required, on average, 75% more blocking drug [4].

Animal studies that have examined the effects of tubocurarine in the presence of glucocorticoids have demonstrated resistance to the effects of the neuromuscular blocker in animals treated with prednisolone [8], dexamethasone [9], betamethasone [10] and triamcinolone [11, 12].

In this study we have demonstrated that the reduction in twitch tension caused by vecuronium was reduced considerably in the presence of betamethasone 1 μmol litre^{-1}. Although the mechanism of this interaction is not fully understood, previous work suggests several possible sites where steroids may affect neuromuscular transmission:

(1) *Motor neurone*

Glucocorticoids have been shown to have a direct facilitatory effect at the impulse generating end of the motor nerve axon. In the cat, administration of large doses of methylprednisolone (8 mg kg^{-1} day^{-1} i.m.) for 1 week altered the electrical properties of spinal motor neurones and increased the excitability of the initial axon segment, the site of physiological impulse initiation [13].

(2) *The presynaptic nerve terminal*

Experimental work suggests that corticosteroids act presynaptically stimulating the synthesis, spontaneous release and stimulated release of acetylcholine.

(a) *Synthesis of acetylcholine.* The synthesis of acetylcholine is related directly to the supply of extraneural choline, which is transported by a sodium dependent carrier system located at the presynaptic membrane of the nerve terminal. Hemicholinium-3 is a presynaptic inhibitor of this carrier-mediated transport and virtually abolishes the synthesis of acetylcholine. Dexamethasone and prednisolone partially antagonize hemicholinium-3 [14] and increase acetylcholine synthesis *in vitro* by facilitating the uptake of choline and its subsequent incorporation into acetylcholine [15]. Electron micrographs of the neuromuscular junction support corticosteroid enhanced acetylcholine synthesis by revealing increases in the mean size of synaptic vesicles after incubation with prednisolone and dexamethasone [16].

(b) *Spontaneous release of acetylcholine.* Micromolar concentrations of prednisolone and dexamethasone have been shown to increase both the amplitude and frequency [17–19] of miniature end-plate potentials. These observations suggest that corticosteroids facilitate spontaneous release of acetylcholine.

(c) *Stimulated release of acetylcholine.* The application of small concentrations of prednisolone (1 μmol litre^{-1}) to the frog neuromuscular junction results in a doubling of nerve evoked end-plate currents. However, if prednisolone is added specifically to the end-plate via a micropipette,

such a response is not observed. This implies that stimulated release of acetylcholine may be augmented by corticosteroids acting at a presynaptic site [20].

(3) *Postsynaptic nerve terminal*

In the rat, large concentrations of prednisolone (0.6 mmol litre^{-1}) have a depressant effect on neuromuscular transmission. However, dexamethasone counteracts the suppression of contraction caused by tubocurarine at small concentrations, but this effect is not observed when greater concentrations of steroid are used [9]. It has been suggested, therefore, that the presynaptic facilitatory effects of small concentrations of corticosteroids on acetylcholine release may be overcome by a greater postsynaptic depressant effect at large concentrations [21]. This may explain why some workers have failed to demonstrate any evidence of recovery from tubocurarine after administration of prednisolone [22].

The evidence suggests that the major site of the facilitatory actions of corticosteroids at the neuromuscular junction is prejunctional and it seems feasible that the attenuation of competitive neuromuscular block observed in our study is a consequence of steroid-induced enhancement of acetylcholine synthesis and release.

A prejunctional reduction in release of acetylcholine by vecuronium, in addition to postjunctional receptor block, has been reported using *in vitro* toad neuromuscular preparations [23]. However, conclusions made from *in vitro* neuromuscular preparations investigating presynaptic modulation are limited [24] and the presence of autoregulating presynaptic acetylcholine receptors at the motor end-plate area has been questioned [25]. Perhaps of more clinical relevance are the observations of Baker and his colleagues in anaesthetized cats. They reported a biphasic prejunctional effect of vecuronium; at small concentrations of vecuronium, acetylcholine release was increased, while at greater concentrations acetylcholine release was reduced [26]. This may indicate that vecuronium interacts with two distinct populations of presynaptic acetylcholine receptors [24] or that the *in situ* effects of cholinergic antagonists (or agonists) on neuromuscular transmission are mediated, in part, by the central nervous system [25]. Whether the interaction between vecuronium and betamethasone occurs at presynaptic cholinergic receptors has not been demonstrated in this report, but it would be of interest to examine further the interaction at the neuromuscular junction between corticosteroids and other cholinergic agonists–antagonists in terms of pre- and postjunctional effects.

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Trichinella pseudospiralis in humans: description of a case and its treatment*

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Abstract

The first known human case of *Trichinella pseudospiralis* myositis is described. A 33 years old woman reported 5 years of relatively mild symptoms of tiredness, muscle fatigue and muscle pain after exercise. She had minimal proximal weakness. Creatinine kinase was significantly elevated, and muscle biopsy showed polymyositis and *Trichinella* larvae. Steroid treatment dramatically worsened the weakness. Treatment with albendazole led to complete resolution of symptoms and laboratory abnormalities. Diagnosis and identification of the parasite were based on the distinctive appearance of the unencapsulated larvae and their movement in fresh muscle, plus clinical and laboratory findings.

Introduction

Trichinella pseudospiralis was described by GARKAVI (1972) from the raccoon (*Procyon lotor*), and is characterized principally by the lack of encapsulation of its larval stage, allowing freedom of larval movement in and between the muscle cells. Recent opinion very strongly favours the separation of *Trichinella* into 5 species (POZIO *et al.*, 1992), of which *T. pseudospiralis* is the only one that has not previously been recorded from humans. Successful experimental infections in monkeys have supported the likelihood that *T. pseudospiralis* infection might be as dangerous to humans as the classical *T. spiralis* infection (PAWLOWSKI & RUITENBERG, 1978; TEPPEMA *et al.*, 1981).

Although *T. pseudospiralis* has a sylvatic life cycle favouring small wild predators, rodents, and raptorial birds as its hosts, domestic pigs have been successfully infected experimentally, thus suggesting a more accessible route by which humans could become infected (GARKAVI, 1972; PAWLOWSKI & RUITENBERG, 1978; OBENDORF *et al.*, 1990).

Thus it seemed that eventually a human case would be discovered, and notice of the first such case was given recently (ANDREWS *et al.*, 1993). The present paper describes more fully the characteristics of this infection and its treatment.

All 5 known species of *Trichinella* have now been found in humans, with each species presenting a different clinical picture (POZIO *et al.*, 1992). This, together with the identification of species-distinctive geographical distributions and life cycles (CAMPBELL, 1988), makes it necessary to distinguish the various disease types at present embraced by the generic term 'trichinosis'. Therefore, in the interests of clarity, we have adopted the terms 'spiralis-trichinosis' and 'pseudospiralis-trichinosis' in this paper.

Case report

The patient was a 33 years old physically fit English woman. She carried out botanical fieldwork in many European countries, Indonesia, and Kenya up to 1979. During 1980–1985 she worked in Australia, mainly Tasmania, but briefly visited other Australian states. Following a visit to the United Kingdom in mid July 1985 she moved to Dunedin, New Zealand, in September. A year later she first sought medical attention because of episodes of racing heart and palpitations, which lasted several weeks and interfered with her sleep. Examination showed no tachycardia. Routine blood tests (28 October 1986) showed a mild disturbance of transaminases and mild eosinophilia (Table). During 1987–1988 she worked in the Northern Territory of Australia, where, in the tropical heat, she began to have fluctuating abnormal physical tiredness, and debilitating mental fatigue with peaks 2 or 3 times per year lasting several days.

She next sought medical advice in Wellington, New Zealand (11 August 1989), again complaining of a racing heart and tiredness. The transaminases were elevated, and a glandular fever-like illness was suspected. The transaminases were still elevated in 1990, but other indices of liver function (albumin and prothrombin time) were normal. The eosinophil count was also normal.

Liver biopsy in 1990 showed mild periportal inflammation. Low-dose prednisone for presumed chronic hepatitis was begun. While visiting the United Kingdom she consulted a gastroenterologist. Her creatinine kinase (CK) level was found to be significantly elevated (3253

Table. Selected haematological data for a patient infected with *Trichinella pseudospiralis*, 1986 to 1992

Date[a]	Aminotransferases Aspartate (5–40 iu/L)	Aminotransferases Alanine (5–50 iu/L)	Creatinine kinase (15–150 iu/L)	Alkaline phosphatase (20–200 iu/L)	Bilirubin (2–20 μmol/L)	Eosinophils ($0–0·5 \times 10^9$/L)
28.10.86	82	99	–	34	13	$0·8 \times 10^9$
11.8.89	138	136	–	–	9	$0·2 \times 10^9$
4.4.90	92	92	–	–	9	0
19.6.90	186	191	–	31	11	0
8.8.90	–	1145	–	–	48	–
29.4.91	225	269	3253	–	7	–
17.6.91	228	324	5436	–	–	$0·47 \times 10^9$
17.7.91	–	–	2168	–	–	–
30.10.91	–	298	3054	–	7	–
24.1.92	–	–	5532	–	–	–
2.11.92 (Anthelmintic treatment)	143	134	3294	–	12	–
4.11.92	–	–	1450	–	–	–
16.11.92	–	–	385	–	–	–
23.11.92	–	–	142	–	–	–
4.12.92	–	–	141	–	–	–

[a]Day.month.year.
[b]Normal range and units in parentheses.

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iu/L), and the steroids were stopped. Once attention was directed to a possible muscle problem she recollected easy tiring of leg muscles on exertion and intermittent aches in the quadriceps and calves lasting several days. Similar symptoms had occurred in her biceps and forearms. By the time of her return to New Zealand she had developed some slight swallowing problems and, for the first time, fatigue walking on the flat.

Further investigations (17 June 1991), performed in Dunedin, New Zealand, included an electromyograph, which showed fibrillations and small polyphasic units suggestive of myositis. A deltoid muscle biopsy showed polymyositis. CK levels had increased (5436 iu/L).

On return to Wellington she was referred to one of the authors (D.A.) for treatment. Clinical examination showed minimal weakness of shoulder abduction, but was otherwise normal. Prednisone, 40 mg/d, was begun for the myositis (27 June 1991). After an initial improvement, weakness increased, reaching a point where she was unable to squat or perform a sit-up, and she had increasing difficulty swallowing. Myasthenia gravis was considered and prednisone was reduced to 20 mg/d.

However, a Tensilon® test and rapid repetitive stimulation on 2 October 1991 were negative. The prednisone was increased to 80 mg/d (8 October 1991), with little change. Azathioprine was begun (30 October 1991), but one month later it was clear that immunosuppressive therapy had unaccountably worsened her condition. All treatment was stopped and a few days later she developed a hot, red, raised, itchy rash over her face, neck, and upper body, which lasted 10 d. Two months after stopping treatment she had no significant weakness.

Muscle biopsy review showed probable *Trichinella spiralis* larvae, in addition to myositis. The worsening after steroids suggested an infectious process, and the biopsy was referred to 2 of the authors of this paper (J.R.H.A. and R.A.), who noted the elongated form of the larvae and their lack of encapsulation. Larval motion through the muscle cytoplasm at the time of fixation was inferred. These features led the authors, in consultation with Dr D. A. Denham (personal communication), to suspect pseudospiralis-trichinosis. A further muscle biopsy, examined fresh, revealed unencapsulated mobile larvae, and confirmed the diagnosis.

On the basis of this diagnosis the patient was admitted to hospital (2 November 1992) and given albendazole, 400 mg/d and prednisone, 40 mg/d. After 2 d the prednisone was discontinued. On 5 November 1992 the albendazole was increased to 800 mg/d, taken in 2 doses. This treatment continued for 4 weeks, followed by a gap of one week, then recommenced for a further month. The patient responded rapidly to treatment, with CK levels declining sharply to normal (Table). Although she reported a return to a feeling of well-being, she experienced some ache in her leg muscles for at least 4 months following treatment. No side effect of albendazole was noticed or reported.

Materials and Methods

Muscle tissue from the first biopsy (18 June 1991) was sectioned by the Pathology Laboratory, Dunedin Hospital, New Zealand, and sections were stained with haematoxylin and eosin or with trichrome. We examined fresh muscle tissue from the second biopsy (8 October 1992) by teasing fibres in ResDel® mammalian saline, followed by examination under a compound microscope for *Trichinella* larvae. Other tissue from this second biopsy was sectioned by the Pathology Laboratory, Wellington Hospital, New Zealand, and stained with periodic acid–Schiff reagent (PAS), PAS-diastase, β-nicotinamide adenine dinucleotide (reduced form; NADH), adenosine triphosphatase, Gomori trichrome stain, or haematoxylin and eosin.

Laboratory findings and Pathology

The muscle tissue taken by biopsy (18 June 1991) showed active myositis, and contained intracellular parasites in transverse section (18–25 μm diameter) and oblique section (50–150×25 μm). The parasites contained sections of immature reproductive tissue, as well as gut, indicating that they were well developed larval stages. The parasites contained well developed reproductive tissue and, in transverse section, some loosely compacted brown granular haematin-like material. None of the parasites was encapsulated.

Fresh muscle tissue taken from the second biopsy (8 October 1992) contained motile worms, 300–500 μm long, at an estimated density of 16–20 worms per gram of muscle. The movement of the worms was similar to that described by KARMI & FAUBERT (1981) for *T. pseudospiralis* larvae.

In whole specimens the haematin-like material extended for approximately three-quarters of the worm's length. Certain aspects of the morphology, including the state of development, were thought to be atypical of *Trichinella* larvae (Ooi & Van Knapen, personal communication). However, an enzyme-linked immunosorbent assay on 27 August 1992 gave a positive result for *Trichinella* (optical density 0·403; positivity threshold ≥0·400; CDC, Atlanta, Georgia, USA) and deoxyribonucleic acid analysis and Western blotting, currently being carried out, strongly support the identification as *T. pseudospiralis*.

Raised levels of the non-specific enzymes aspartate and alanine aminotransferases and the specific muscle enzyme CK, as found in the present case (Table), are well known in trichinosis (POZNANSKA *et al.*, 1981). The levels of CK were very high (up to 5532 iu/L), and can be compared with an average level for spiralis-trichinosis of 232 iu/L (STUMPF *et al.*, 1981). In spite of fluctuations in CK level over time there was no overall trend indicating any decline in larval activity, or change in activity resulting from the administration of prednisone, although clinical symptoms intensified substantially during this time. High CK levels resulting from persistent larval movement within the muscle cell may be helpful in differential diagnosis.

The muscles appear to be the only source of increased enzyme activity in trichinosis, with neither the liver nor the parasite contributing to any marked degree (POZNANSKA *et al.*, 1981). However, a study of *T. pseudospiralis* in mice (GABRYEL *et al.*, 1981) found that changes occurred in the kidney and liver with the reaction of the liver being regarded as a non-specific hepatitis—one of the apparent symptoms of the case reported here.

The serum albumin level was normal in our case, consistent with the findings of KOCIECKA *et al.* (1981b), who suggested that this feature was useful in differential diagnosis. *T. spiralis* infections characteristically present a marked reduction in serum albumin.

Eosinophilia was found to develop rapidly in monkeys infected with *T. pseudospiralis* and to reach a peak in a little over 3 weeks, gradually declining to low levels by 100 d after infection (KOCIECKA *et al.*, 1981b). In the present case only one raised eosinophil count was recorded, followed by a period when no record was made or the levels were normal.

The sustained presence of larvae in host muscle suggests an ineffectual immune response on the part of the patient. In the present case the inflammatory response revealed by tissue sections appeared relatively weak, a feature of pseudospiralis-trichinosis also seen in monkeys (TEPPEMA *et al.*, 1981). This contrasts with the marked inflammatory response to spiralis-trichinosis.

Clinical symptoms

The symptoms recorded here were those of the muscular phase of the disease. The intestinal phase is of relatively short duration and is self-limiting, and may have been relatively asymptomatic. We concluded that this phase had terminated some time before the patient first sought medical advice.

Experimental infection of monkeys demonstrated expulsion of adult *T. pseudospiralis* about 24 d after infection (KOCIECKA *et al.*, 1981a), but a high number of larvae in muscle was maintained for the duration of the experiment (6 months). In the present case the period of larval viability has been extended to an estimated 7–9 years, with continuing symptoms of their presence. This compares with *T. spiralis* infections in monkeys, in which there is usually a substantial reduction in larval numbers after a few months and consequent loss of symptoms (KOCIECKA *et al.*, 1981a; CAMPBELL, 1983). In other hosts (e.g., polar bears) encapsulated larvae of *Trichinella* can last for many years (D. A. Denham, personal communication). The level of 16–20 larvae per gram of muscle found from the patient suggests that relatively low numbers are capable of causing clinical symptoms. These figures are more or less similar to those of *T. spiralis*, but are considerably less than the clinically important levels of other species (POZIO *et al.*, 1992).

The favoured location for *T. pseudospiralis* larvae in monkeys was the masseter muscle, a feature shared with *T. spiralis*, with the tongue being less favoured by the former (KOCKIECKA *et al.*, 1981a). Swallowing difficulties in the present case appear to have been related to involvement of these muscles, although the patient complained of this symptom only during steroid treatment. The quadriceps and biceps muscles, which this patient referred to as being weaker than expected and aching after exercise, were also among the more highly favoured sites in the monkey hosts.

Episodes of racing heart and palpitations during the earlier phases of the disease may relate to heart muscle involvement. *T. spiralis* is known to enter heart muscle with various consequent cardiac symptoms, including tachycardia.

Periorbital and other facial oedema is a common symptom of spiralis-trichinosis and pseudospiralis-trichinosis (KOCIECKA *et al.*, 1981b). Although the patient did not complain of this, several of her colleagues and friends referred to a puffy appearance around the eyes and face that disappeared after anthelmintic treatment.

In summary, pseudospiralis-trichinosis exhibits the same protean manifestations as spiralis-trichinosis, but with a few significant differences. The delay in seeking consultation after the first recalled symptoms (1–2·5 years) suggests that symptoms in the early stages of the disease in physically fit persons are relatively mild and non-specific.

Source of the infection

Symptoms of pseudospiralis-trichinosis can be expected to arise some 3 weeks after infection (KOCIECKA *et al.*, 1981b). In the case described the patient recalled the first uncharacteristic bouts of tiredness occurring in Australia during 1984–1985 when, apart from a few months on the mainland, she was living in Tasmania. A short period in the United Kingdom preceded her arrival in New Zealand in September 1985, and almost a year passed before she sought the consultation referred to above, the palpitations having begun 2–3 months earlier. Field work in New Zealand in the summer of 1985–1986 gave rise to periods of unusual fatigue, but she maintained a generally high level of activity.

T. pseudospiralis has an erratic distribution, possibly as a result of its having avian co-hosts. Originally described from the raccoon, it has since been found in carrion-feeding birds from Russia and North America, and from quolls (*Dasyurus viverrinus*), Tasmanian devils (*Sarcophilus harrisii*), and a brush-tailed opossum (*Trichosurus vulpecula*) in Tasmania (OBENDORF *et al.*, 1990). There are also records from Spain and India (POZIO *et al.*, 1989). There is no record of *T. pseudospiralis* from New Zealand or mainland Australia, although 4 men in mainland Australia were recently discovered to be seropositive for *Trichinella*, with no firm species identification (P. J. McDonald, personal communication). Infrequent but persistent reports of *T. spiralis* have been made in New Zealand (CAIRNS, 1966; MASON, 1978), with no suggestion of misidentification of larvae. In the present case, linking the onset of symptoms to geographical locality is difficult as the patient travelled widely. Although the known distribution of *T. pseudospiralis* points to Tasmania as the likely source of her infection, mainland Australia, New Zealand, and even possibly the United Kingdom cannot be entirely eliminated.

Until early 1984 the patient was a vegetarian, but then adopted a limited (in terms of quantity) meat diet. This covered the normal range of domestic meats, including pork, and an experiment in 1984 with Tasmanian wallaby that was apparently well cooked. OBENDORF *et al.* (1990) speculated that some wallaby species could act as hosts, and found that pigs could be infected by means of Tasmanian devil and quoll flesh containing *T. pseudospiralis*, but they were not regarded as ideal hosts. This conclusion is consistent with other reports of a low reproductive capacity index for *T. pseudospiralis* in pigs (POZIO *et al.*, 1992). In spite of these reservations, pork is a possible source of infection. In the course of research the patient frequently handled faecal pellets from wallabies, kangaroos and, to a lesser extent, quolls and Tasmanian devils. Larval *Trichinella* transmission via faecal contamination has been suggested (FAUST *et al.*, 1970), but is regarded as unlikely (D. A. Denham, personal communication).

The reason for an apparently recent appearance of *T. pseudospiralis* in the southern hemisphere can only be speculated upon, but migrating carrion-eating seabirds could be responsible.

The information recorded above has led us to the following tentative conclusions: the patient was infected some time between early 1984 and mid-1985, in Tasmania, possibly as a result of eating infected wallaby meat, pork, or pork products, with faecal contamination being, at this stage, the less likely option. A survey of 1768 Tasmanian pigs has, however, proved negative (F. B. Ryan, personal communication).

Treatment

Traditionally, trichinosis has been difficult to treat, with the choice of treatment varying according to clinical severity and the strain or species of *Trichinella* involved. In the present case it was assumed that the adult worms of the intestinal phase had long since been expelled and that treatment should be directed at the larvae of the muscular phase.

A mild or light case of spiralis-trichinosis at a late stage of the disease might call for little more than symptomatic treatment, with the self-limiting nature of the infection eventually ensuring a complete recovery. The use of larvicidal drugs is not recommended in such cases unless there are unusual circumstances (CAMPBELL, 1983).

In the present case the persistence of larvae in the muscle and the associated symptoms called for the use of a larvicidal drug. Mebendazole has largely replaced thiabendazole as the drug of choice in view of its activity against adult worms and both encapsulated and unencapsulated larvae. However, mebendazole and its derivative flubendazole are not well absorbed through the intestine, thus limiting their effects on extraintestinal stages. A trial of albendazole in an outbreak of human trichinosis gave more favourable results, with respect to residual larval infection, than did thiabendazole and flubendazole (FOURESTIÉ *et al.*, 1988). Also, an earlier study had indicated that albendazole was extremely well tolerated (SAIMOT *et al.*, 1983). This information led to the choice of albendazole for treatment of the present case. The results reported here indicate that albendazole is effective against *T. pseudospiralis* in humans, and in this case there was no apparent side effect.

The effect of the drug was monitored through CK levels, and further biopsies were not taken. The patient continued to experience ache in the leg muscles following normal activities, but apparently this phenomenon is not

uncommon following successful treatment of spiralis-trichinosis (CAMPBELL, 1983). The possibility of anaphylaxis as a result of numbers of larvae dying in muscle tissue was guarded against by the use of steroids during the initial stages of anthelmintic treatment.

Conclusions

T. pseudospiralis is capable of producing clinical symptoms in humans. Indications, gleaned from a single case, are that disease caused by this agent could be serious if the initial larval intake was high. However, the opportunities for human infection may be relatively infrequent, as the normal wild hosts of this species have limited contact with humans and are not normally consumed as meat. Also, the low reproductive capacity index for *T. pseudospiralis* in pigs may result in a low frequency and intensity of clinical symptoms acquired by consuming infected pork. On the other hand, infected meat may escape detection during routine abbatoir screening, particularly when the trichinoscopy technique is used, because of the low intensity of the larvae in muscle and their free-moving nature. The possibility, however remote, of infection via faecal contamination suggests a need for further investigation to place this issue beyond doubt. The potential for pseudospiralis-trichinosis to become widespread is reasonably high, considering the presence of avian hosts and the ability of the worm to infect rats and pigs. This is of particular public health concern for countries that are unfamiliar with any form of trichinosis, and where the disease might escape detection. The possibility of carrion-eating seabirds being carriers should be investigated.

Although many of the features of pseudospiralis-trichinosis are similar to those of the spiralis form of the disease, differential diagnosis can be made on the observation of persistent and chronic elevated CK levels and muscle symptoms, plus motile unencapsulated larvae in muscle biopsy. When the parasites are not seen on biopsy and polymyositis is diagnosed, worsening of the disease following steroid treatment should alert clinicians to the possibility of pseudospiralis-trichinosis.

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Short Report

Human intestinal capillariasis: first report from India

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The first documented case of human intestinal capillariasis was reported from the Philippines (Chitwood *et al.*, 1968). Subsequently, it has been reported from Thailand, Japan, Taiwan, Iran, Egypt, (Cross, 1992) and the United Arab Emirates (El Hassan & Mikhail, 1992). This report of the parasite in India indicates that it may have a wider distribution in Asia than suspected earlier.

Case report

A 45 years old clerk from Jamshedpur in central India, with no history of international travel, presented with intermittent abdominal pain, borborygmi, loss of weight (8 kg) and appetite, and generalized weakness for one year. He had not had diarrhoea, nausea or vomiting, but had been treated with mebendazole syrup 100 mg twice a day intermittently, for a total period of 3 months during the previous year. While taking mebendazole, his abdominal pain decreased in frequency and intensity, but it recurred on stopping treatment. He also had bronchial asthma, for which he had swallowed whole raw fish as part of an indigenous medical therapeutic regimen at Puri, a seaside resort and pilgrimage centre, where fish are available both from the sea and from Chilka, a large brackish water lake nearby.

On examination, he was a thin, middle-aged man weighing 42 kg, with no significant finding except bilateral rhonchi and a few basal crepitations. Laboratory investigations were normal, except for a low haemoglobin level (9·7 g/dL) and an erythrocyte sedimentation rate of 24 mm at 1 h. Examination of the stool did not reveal any parasites.

Fig. 1. Jejunal biopsy showing invasion by adult worms (arrows) (×45).

Review of a barium meal examination done elsewhere showed diffuse involvement of the small bowel with loss of the normal mucosal pattern, narrowing of the lumen and separation of the bowel loops. Based on these findings a jejunal biopsy was done. The jejunal aspirate showed an adult male *Capillaria philippinensis*, measuring 2·9 mm with a sheathed spicule 0·3 mm long, a female third stage larva with an oral spear and peanut shaped ova, measuring 0·020–0·021×0·041–0·045 mm, bi-operculated with flattened plugs. Some ova were embryonated. The biopsy showed marked villous atrophy, crypt effacement, diffuse infiltration of the lamina propria with plasma cells, lymphocytes, eosinophils and polymorphonuclear leucocytes, and invasion by adult worms (Fig. 1). Electron microscopy showed the adult worm in epithelial tunnels but not penetrating the basal lamina. The cells in direct contact with the worm showed evidence of degeneration, with swollen mitochondria and distended rough endoplasmic reticulum (Fig. 2). The morphological identification as *C. philippinensis* was confirmed by the Liverpool School of Tropical Medicine, UK.

Based on the biopsy and aspirate findings, the patient was treated with 400 mg of thiabendazole 3 times a day for 3 weeks. After completion of the anthelmintic therapy, a second jejunal biopsy showed reversion of the earlier change to normal. No parasite was found in the biopsy, jejunal aspirate or stool at this time.

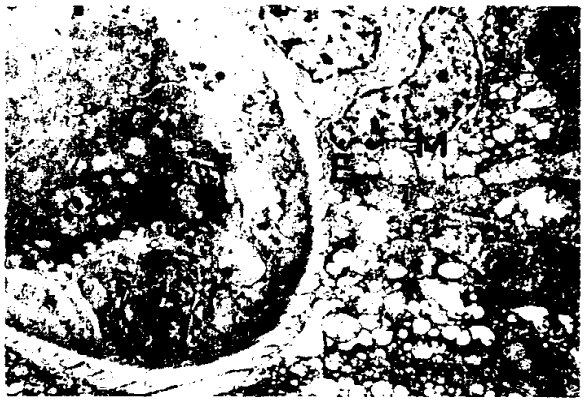

Fig. 2. Electron micrograph of section of jejunum. Adult *Capillaria* (C) appears to lie in direct contact with an epithelial cell cytoplasm (E) with swollen mitochondria (M) (×8000).

Discussion

This is the first case report of *C. philippinensis* from India. Usually, only patients with *Capillaria* eggs in their stool have the characteristic clinical features of the disease. The fact that this patient did not have diarrhoea, and no egg was seen on stool examination, could be due to his intermittent treatment with an adequate dose of mebendazole.

Human intestinal capillariasis is usually transmitted by consumption of raw fish. This patient did give a history of ingestion of raw fish, although it was not routinely part of his diet.

Pisciculture is being increasingly practised in India and the parasite may have been introduced with fish imported to start fish farms, or by migratory fish-eating birds. With multiple reports of intestinal capillariasis to the east and west of the Indian subcontinent, it seems likely that the parasite has a wide distribution in Asia. Capillariasis should therefore be part of the differential diagnosis of a patient with obscure abdominal symptoms or abnormal intestinal biopsy appearance in India.

Acknowledgements

We thank Mrs Wendi Bailey of the Liverpool School of Tropical Medicine for confirming the identity of the parasite and Dr A. K. Bandhopadhyay for providing further information about the patient.

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ORIGINAL PAPER

K. D. Clements · D. Rees

Preservation of inherent contractility in isolated gut segments from herbivorous and carnivorous marine fish

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Abstract Patterns of inherent contractility were investigated in isolated gut segments from three species of New Zealand temperate-water labroid fish: the herbivorous butterfish *Odax pullus* (Family Odacidae), and the carnivorous banded wrasse *Notolabrus fucicola* and spotty *Notolabrus celidotus* (Family Labridae). To maintain the functional viability of gut tissue over extended periods, physiological solutions were formulated for each genus based on biochemical analyses of plasma constituents. A Res-Del perfusion system was used to maintain isolated gut segments under conditions of constant pH, temperature, and laminar flow dynamics. Gut segments exhibited spontaneous contractility over periods of days. Segments from *N. fucicola* and *N. celidotus* were continuously active, whereas segments from *O. pullus* showed discontinuous activity with evidence of daily rhythmicity. Contractions were slight in segments from *O. pullus* compared to the two *Notolabrus* species. Contractile strength correlated with gut morphology, with circular and longitudinal muscle layers markedly greater in extent in the *Notolabrus* species than in *O. pullus*. The pattern of gut activity in *O. pullus* is possibly associated with the retention of algal material in the intestine for lengthy periods. This hypothesis is supported by the daily feeding pattern of this species, and the probable involvement of fermentation in its digestive process.

Abbreviations *RS-I* Rees-Simpson inotropic physiological solution · *RS-F* physiological solution for fish · *RS-F$_o$* physiological solution for *Odax pullus* · *RS-F$_n$* physiological solution for *Notolabrus* spp.

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Introduction

The mechanisms involved in the digestion and assimilation of plant material by terrestrial herbivorous vertebrates have been well studied (Stevens and Hume 1995). In contrast, the digestive mechanisms of marine herbivorous fish, the dominant group of herbivorous vertebrates in the sea, are poorly understood (Horn 1989; Choat 1991). Previous workers have described a variety of digestive mechanisms in marine herbivorous fishes at the anatomical level (reviewed in Lobel 1981; Horn 1989; Choat 1991), but to date there has been little research at the biochemical or physiological level. This situation is partly the result of geography: marine herbivorous fishes are distributed predominantly in the tropics or the southern hemisphere, and hence are not readily accessible to most biochemists and physiologists. We investigated aspects of gut physiology in three closely related Australasian labroid fish species: the butterfish *Odax pullus*, a herbivorous member of the temperate-water family Odacidae, and two carnivorous species of the family Labridae, the banded wrasse *Notolabrus fucicola* and the spotty *Notolabrus celidotus*.

Previous studies of fish physiology have employed in vitro techniques to investigate factors such as gut activity (Burnstock 1958), gill physiology (Perry et al. 1984), intestinal absorption (Bogé et al. 1981; Buddington et al. 1987), electrophysiology of skeletal muscle fibres (Hudson 1968), and heart metabolism (Driedzic and Hart 1984). The maintenance of tissue viability, in terms of biochemical and physiological function, is critical to the demonstration of any experimentally induced effect using in vitro tissue preparations. In practice, the degree of viability of isolated nerve/muscle preparations is largely dependent upon the artificial serum solution or saline used. The design and use of physiological solutions was reviewed by Burton (1975) and Rees (1989a), who point out that substantial deficiencies exist in the plethora of solutions currently used. A prerequisite for in vitro investigations on isolated gut

preparations from herbivorous fishes, therefore, was to establish a physiological medium that would be more reflective and supportive of the in situ biochemical and physiological processes of these tissues.

The design of Rees-Simpson inotropic (RS-I) mammalian physiological solution (Rees 1978) was based upon levels of ionic species (Pederson 1973; Rees 1989a) in human, rat and mouse sera. RS-I provides a suitable nutritive, non-phosphate buffered environment, and thereby differs from previous phosphate-buffered 'Ringer' solutions. The longevity of isolated tissue-organ preparations from the mouse, rat, guinea pig and rabbit is 1–10 days in RS-I (Rees 1989b). We used this technology as a basis to investigate patterns of gut activity in *O. pullus* and the *Notolabrus* species.

O. pullus is one of three herbivorous species in the Odacidae (Gomon and Paxton 1985). It is endemic to New Zealand, where it is more abundant and attains a larger size in the southern part of its distribution (Ayling and Cox 1982). *O. pullus* lacks a stomach, has a simple gut with a relative gut length (total gut length divided by standard length of fish) of about 1.5 (Clements and Bellwood 1988), and harbours a diverse, gut-resident endosymbiotic microbiota (Clements 1991). The diet of adult *O. pullus* consists of fucoid and laminarian macroalgae (Choat and Clements 1992; Clements and Choat 1993), with maximum feeding activity in the immediate post-dawn period (Choat and Clements 1993). There is no indication of a tidal rhythmicity in feeding rate in this species, although tidal state is known to influence diet composition (Clements and Choat 1993). *O. pullus* is active only during daylight hours, and individuals retire to slumber sites at nightfall (Choat and Clements 1993).

N. fucicola occurs in New Zealand and southeastern Australia, while *N. celidotus* is endemic to the New Zealand region (Ayling and Cox 1982). Both *Notolabrus* species feed during daylight hours on a wide range of benthic invertebrates, and are inactive at night (Francis 1988).

Muscular activity of the gut is responsible for the transit of food through the alimentary tract at an optimal rate for digestion and absorption (Stevens and Hume 1995). Furthermore, it has been suggested that lengthy gut transit times are a prerequisite for intestinal fermentation in vertebrates (Horn 1989). It was thus of interest to determine whether patterns of gut contractility differed between the herbivorous hindgut-fermenter *O. pullus* and the carnivorous *Notolabrus* species. Given the daily periodicity of feeding in *O. pullus*, a requirement for this study was the maintenance of gut tissue in vitro for periods of at least 24 h. The aims of this study, therefore, were: (1) to modify a mammalian physiological solution, RS-I, to accommodate isolated *O. pullus* gut preparations, (2) to examine gut contractility of this species in vitro over extended periods of time (1–5 days), and (3) to compare these results with gut preparations from two related, carnivorous fish species, *N. fucicola* and *N. celidotus*.

Materials and methods

Collection of specimens

All fish used in this study were collected near Wellington, New Zealand (41°18′S, 174°48′E), in May and June 1991. *O. pullus* were captured by speargun from subtidal reefs. Both *Notolabrus* species examined occur in the same subtidal habitats as *O. pullus*, although unlike *O. pullus* both are carnivorous. *N. celidotus* used for blood analyses were captured by otter trawl, while gut tissue from this species and *N. fucicola* was obtained subsequently from fish collected by spear or hook and line. All fish were taken immediately from the water following capture, and killed by a blow to the head. Blood sampling and dissection of gut tissue were conducted immediately after capture. In fish used for blood sampling, the pericardial cavity was exposed by dissection and blood removed directly from the bulbus arteriosus with a 23 gauge needle and syringe. Blood samples (1–2 ml) were then placed into sealed (Microtainer) heparinised plasma separator tubes and transported on ice to the Division of Chemical Pathology, Wellington Hospital, for analysis. These tubes were then centrifuged and the plasma retained for analysis.

Biochemical analyses of plasma

Biochemical analyses of plasma samples were carried out using a Hitachi Boehringer Mannheim 717 chemical analyser using standard clinical procedures. Osmolality was measured using the freezing point depression method on a Fiske "One Ten" osmometer. Plasma pH was measured with an ion selective electrode on an AVL995 blood gas analyser. Plasma analyses were performed on 11 samples from *O. pullus* and 3 samples from *N. celidotus*, with one of the *N. celidotus* samples analysed being pooled from 10 fish. The results of these analyses were used to formulate appropriate RS-I solutions for *O. pullus* and the *Notolabrus* species, referred to as RS-F_o and RS-F_n, respectively. RS-F was derived from a consideration of the activity coefficients of individual ionic species (Burton 1975) and the free, ionised levels of calcium and magnesium (Pedersen 1973). The formulation of RS-F_o and RS-F_n differed only in terms of the concentration of sodium ions, potassium ions and osmolality.

In vitro techniques

Fish used as donors of gut tissue were rinsed with the appropriate physiological solution to remove sea water before dissection and removal of the gut segments. *O. pullus* specimens in which the gut cavity was damaged during collection were not used for perfusion. Labroid fishes such as *O. pullus* and the two *Notolabrus* species lack a gastric stomach, and have a simple, relatively undifferentiated intestine (Zihler 1982). The gut anatomy of the three species used in this study was therefore very similar, although the gut in *O. pullus* was longer and wider than in the two labrids. The entire gut was removed from the fish and divided into five sections of equal length in *O. pullus* (referred to as sections I–V from anterior to posterior respectively) and three sections of equal length in the *Notolabrus* species (referred to as sections I–III). Most sections were then immediately cut into segments of approximately 2 cm in length, rinsed with physiological solution to remove gut contents, and placed in sealed, sterile 50-ml vials containing the appropriate carbogenated (i.e. saturated with 95% O_2/5% CO_2) physiological solution (i.e. RS-F_o for *O. pullus*, RS-F_n for *Notolabrus* species). Some gut segments from *O. pullus* were ligated with thread in situ at point of capture to retain gut contents, but were otherwise treated as above.

Vials containing gut tissue were maintained at ambient temperatures of 13–15 °C during a transit time back to the laboratory of less than 2 h. Gut segments from ten *O. pullus*, two *N. celidotus* and two *N. fucicola* specimens were perfused with RS-F solutions in the course of the study. Samples taken at point of capture from

gut segments of two *O. pullus* 240 and 340 mm SL (standard length the distance from the tip of the snout to the end of the vertebral column) and one *N. fucicola* 190 mm SL were immediately fixed in Bouin's solution. These samples were subsequently dehydrated, embedded, transversely sectioned and stained to measure dimensions of the circular and longitudinal muscle layers.

Upon return to the laboratory, gut tissue for perfusion was stored at 8–10 °C in sealed, sterile 50-ml vials in the appropriate RS-F solution that was replaced with fresh, carbogenated RS-F solution every 12 h prior to experimentation. The storage temperature used in this study (8–10 °C) was preferred to lower (i.e. 0–6 °C) temperatures to prevent permanent inactivation of enzyme function through temperature-induced changes in membrane fluidity (McMurchie et al. 1973), plus loss of neuronal (peristaltic) activity originating within the myenteric plexus (Burnstock 1958).

The Res-Del perfusion bath was filled with RS-F solution and the gut segments aligned horizontally and transversely within the perfusion bath (Fig. 1). Fine steel pins were used to secure one end of the gut segments to the Sylgard resin base of the central compartment, with the gut lumen remaining open to ensure access of the perfusion solution in non-ligated segments. The free end of the segments (i.e. the open end of non-ligated segments) was attached to the fine steel hook of the transducer arm of a Grass Ft.03 isometric force-displacement module (Fig. 1). Force-displacement transducers were aligned horizontally to measure the inherent myogenicity and changes in tension of the visceral musculature in the isolated gut segments which were recorded using a Gould RS 3200 oscillograph. Both non-ligated (open) and ligated (closed) gut segments were maintained at a set tension of 1.0 ± 0.3 g. The set tension adopted for each preparation was that which (1) resulted in minimal change (i.e. ± 10%) in the recorded tension over the 15 min equilibration period (see below), or (2) evoked optimal "cold" contracture (Burnstock 1958) or transmural responses (Fänge and Grove 1979).

Isolated gut segments were washed with the RS-F perfusion solutions to remove excess ions and blood, and then perfused with a flow rate of 1–4 ml min^{-1} at 17 ± 0.5 °C (pH 7.12 ± 0.03). A period of equilibration (c. 15 min) was adopted to allow the gut segments to adjust to the serum-simulated ionic/substrate composition of the perfusion solution. At the end of the equilibration period the set tension of all gut segments was checked and re-set, using the vernier control of the force-transducer arm (Fig. 1) to a set tension of 1.0 ± 0.3 g for the duration of each experiment. The functional viability of the neural and muscular components of the isolated gut segments was assessed by two methods. The first method involved injecting cold (8–10 °C) RS-F solution, via a 27 gauge syringe needle, over the submerged surface of the preparation so evoking a "cold contracture" response of the visceral musculature (Burnstock 1958). In the second method, direct stimulating electrodes were placed across the gut preparations (Fig. 1) to deliver 10 V pulses (0.1 ms duration) at 100 Hz for 10 s, thereby evoking transmural or direct current (DC) stimulation of the intrinsic neuronal networks (Fänge and Grove 1979). Irreversible relaxation over time and/or absence of the evoked "cold" or DC responses in these experiments were used to indicate non-viability and the preparation discarded.

Results

Ionic and biochemical analyses of plasma samples

The ionic and biochemical profiles of plasma samples from *O. pullus* and *N. celidotus* are shown in Table 1. The two species were very similar in plasma composition. The only major difference between the species was the level of sodium ions and the concomitant osmolality, both of which were higher in *N. celidotus*. The composition of RS-F_o and RS-F_n solutions formulated on the basis of these plasma results is presented in Table 2.

Time series analyses of isolated gut activity

Gut segments from *N. celidotus* and *N. fucicola* perfused with RS-F_n solution exhibited spontaneous, large (0.9–2.7 g) contractions. Contractions were of short duration (< 12 s) or consisted of trains of 4–7 spikes at a

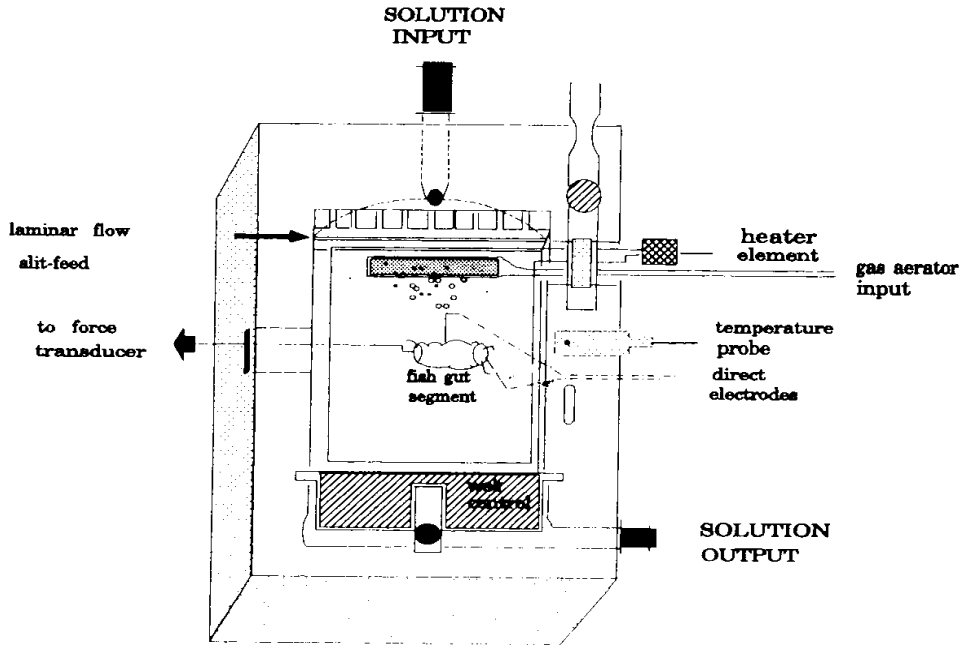

Fig. 1 Diagrammatic representation of the horizontally designed Res-Del perfusion bath seen from above. Isolated gut segments were placed within the central compartment of the bath and were perfused by laminar fluid flow provided by a plenum chamber/slit-fed input module. Control of the fluid temperature was achieved directly within the central compartment of the bath by a temperature sensor IC-probe/heating element system. A sintered glass aerator maximised oxygenation of the perfused isolated preparations which were maintained totally submerged during experimentation. The transparent design of the central compartment within the Res-Del perfusion bath allowed visual (microscopic) examination of gut preparations for manipulative procedures, e.g. electrode placement. Drawn to scale, scale ×0.7

Table 1 Composition of plasma from *Odax pullus* and *Notolabrus celidotus*. Results are mean mM ± SEM. Values in *parentheses* indicate sample size (*NE* not estimated)

Parameter	*O. pullus*	*N. celidotus*
Na^+	184.55 ± 1.12 (11)	212.33 ± 4.68 (3)
K^+	2.10 ± 0.07 (9)	2.73 ± 0.35 (3)
Ca^{2+}	2.93 ± 0.08 (7)	2.92 ± 0.24 (3)
Mg^{2+}	1.73 ± 0.13 (6)	1.40 ± 0.43 (3)
HCO_3	8.58 ± 0.26 (4)	10.13 ± 1.64 (3)
PO_4	3.08 ± 0.14 (7)	5.48 ± 1.13 (3)
D-glucose	3.24 ± 0.08 (6)	6.87 ± 1.14 (3)
Total protein	33.57 ± 2.82 (7)	33.50 ± 5.52 (2)
Osmolality	371.57 ± 5.94 (7)	423.00 ± 10.55 (3)
pH	7.09 ± 0.13 (5)	NE
Urea	0.57 ± 0.17 (6)	2.35 ± 0.45 (3)

Table 2 Composition of RS-F_o physiological solution used to perfuse *O. pullus* gut segments. Values in parentheses are those for RS-F_n, the solution used to perfuse *Notolabrus* gut segments, where RS-F_n differs from RS-F_o. Solutions aerated with carbogen (95% O_2/5% CO_2). pH = 7.12 ± 0.03 at 10–20 °C. Concentrations unless otherwise stated are in mmol/l. (*BES* N,N-*bis*(2-hydroxyethyl)-2-aminoethanesulfonic acid)

Category	Component	Concentration
Salts	NaCl	172.00 (200.00)
	KCl	2.10 (2.80)
	$CaCl_2$	1.30
	$MgCl_2$	1.00
Buffer	$NaHCO_3$	13.00
	BES	1.00
Substrates	D-glucose	10.00
	glycerol	0.11
	Na^+-glutamate	0.30
	L-glutamine	0.40
	Na^+-aspartate	0.02
	DL-carnitine	0.05
	Choline Cl^-	0.01
	TPP (cocarboxylase)	40.00 nmol/l
	Insulin (porcine)	25.00 mIU/l

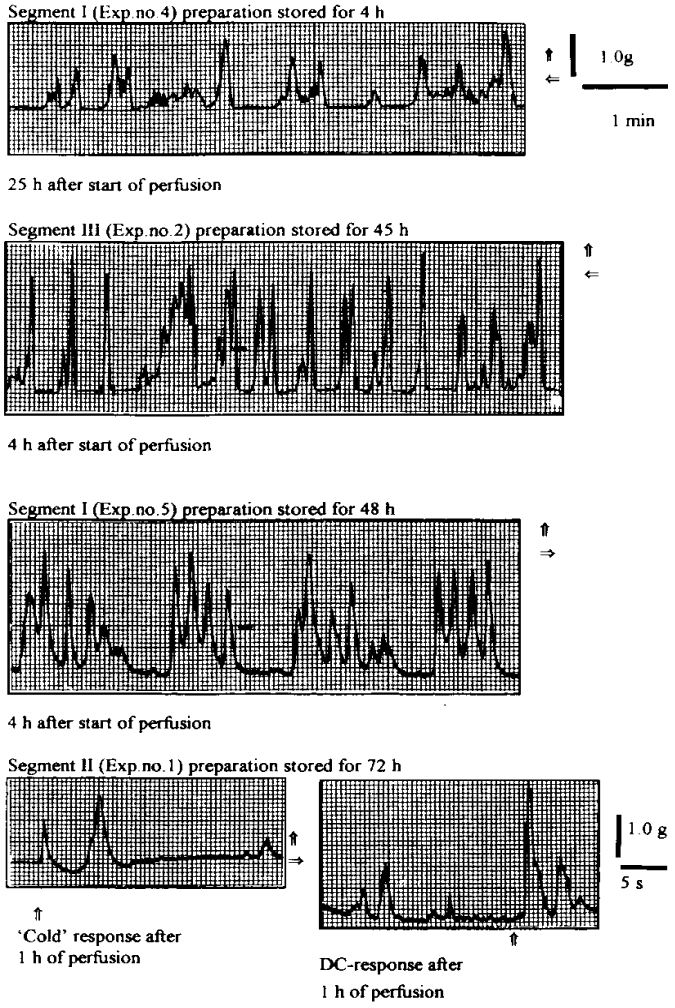

Fig. 2 Spontaneous gut activity recorded from segment I of *Notolabrus fucicola* and segments II and III of *Notolabus celidotus*. *Vertical arrows* indicate direction of contraction, and *horizontal arrows* indicate direction of trace (*DC* direct current)

rate of 60–120 h^{-1}. After storage at 8–10 °C for 4 h (*N. fucicola*) or 45 h (*N. celidotus*), activity at 17 °C was continuous for 43.5 h and 22.5 h, respectively (Fig. 2, Table 3). Similar continuous activity for 8 h (*N. fucicola*) and 7 18 h (*N. celidotus*) was observed in segments perfused after storage for 48 h and 67.5–72 h, respectively (Fig. 2, Table 3).

The activity of isolated non-ligated gut segments from *O. pullus* was very different from that of gut segments from the two *Notolabrus* species. Contractions in isolated gut segments of *O. pullus* occurred at a rate of 2–5 h^{-1}, compared to 60–120 h^{-1} in *N. celidotus* and *N. fucicola* (cf. Figs. 2, 3). The gut activity of *O. pullus* was characterised by either contractures of long duration (6–12 min) followed by rapid relaxation, or single, biphasic spiked contractions lasting 10–50 min (Fig. 3). Preparations from all five segments of *O. pullus* gut showed some degree of activity during perfusion periods of up to 37 h. All eight non-ligated preparations from gut segment III displayed activity, even after storage durations of up to 104 h (see Table 4). In the other non-ligated preparations, activity was observed in one of two segment I preparations, one of two segment II preparations, three of six segment IV preparations, and one of three segment V preparations.

The non-ligated *O. pullus* isolated gut preparations exhibited either no change in tone (tension) or transient phases of altered tonus for the duration of the experiments. Seven preparations (Table 4) showed no discernible contractile activity during perfusion, and simply maintained a constant (±0.03 g) tension. These preparations could have been considered dysfunctional, except that in all instances tested (experiments 3, 5, Table 4) they exhibited evoked contractures to topical application of "cold" RS-F_o (Fig. 3 experiment 12). Gut activity in *O. pullus* preparations often ceased for periods of up to 8–12 h and then spontaneously resumed

Table 3 Experimental protocol and activity profiles for gut segments from *N. celidotus* (*Nc*) and *Notolabrus fucicola* (*Nf*) stored and perfused with RS-F_o (* 2nd day of perfusion, ** 3rd day of perfusion, *k* no change in tension)

Experiment no	Species	Gut Segment	Storage @ 8–10 °C duration (h)	Time set in vitro @ 17 °C	Tension status	Perfusion duration (h)	Time of activity	Activity duration (h)
1	*Nc*	II	72	1500	k	18	1500–2400 0000–0900*	18
2	*Nc*	III	45	1000	k	22.5	1000–2400 0000–0830*	22.5
3	*Nc*	I	67.5	0900	k	7	0900–1618	7
4	*Nf*	I	4	1200	k	43.5	1200–2400 0000–2400* 0000–0730**	43.5
5	*Nf*	I	48	1000	k	8	1000–1800	8

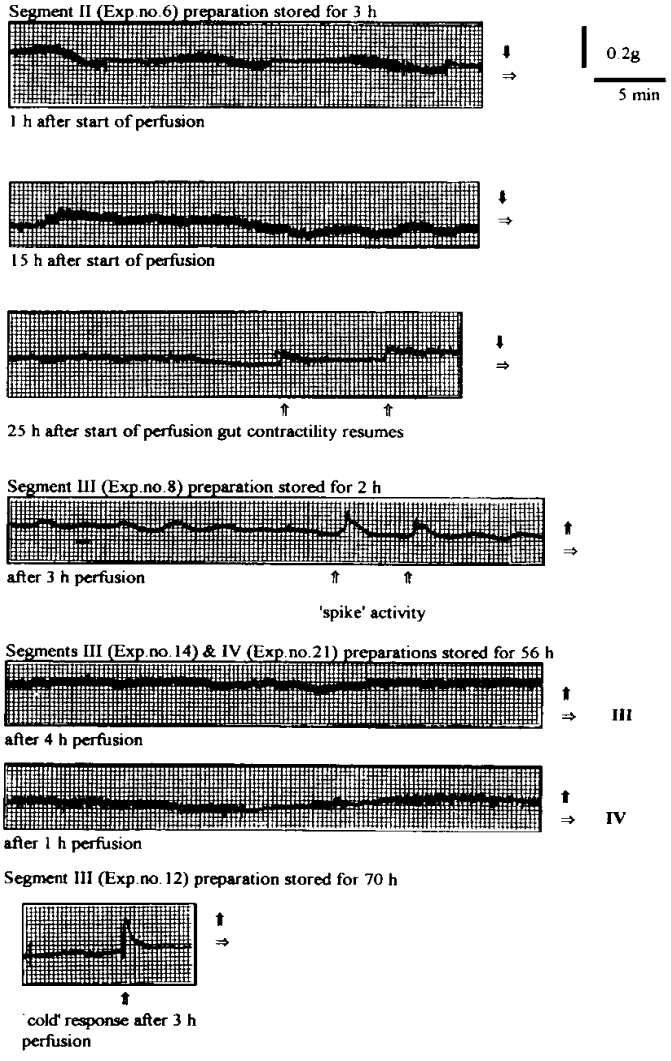

Fig. 3 Gut activity in segments II, III and IV of *Odax pullus*. *Vertical arrows* indicate direction of contraction, and *horizontal arrows* indicate direction of trace

activity as shown for segment II in experiment 6 (Fig. 3, Table 4). Interestingly, in this latter preparation, far greater activity became evident around dawn with the preparation giving a classical "cold" visceral muscle response after 19 h perfusion. Spontaneous activity in this preparation reappeared again virtually 25 h after the commencement of the experiment (Fig. 3, experiment 6).

Previous reports suggest that the myogenic activity of fish gut preparations is related to the degree of distension of the gut wall (Burnstock 1958; Fänge and Grove 1979). We tested this using gut segments I and V from *O. pullus* that were ligated in situ to retain gut contents. Ligated segments of section I were stored in RS-F_o solution for 56 h and 70 h, while ligated segments from section V were established in the perfusion system after storage times of 5 h. The observed activity in the ligated section I segment was initially low and comparable to that observed in non-ligated segments. However, by 0300 hours activity in this preparation was approximately 300% greater than that observed at the commencement of the experiment. The two ligated segments of section V during perfusion periods of 18.5 h and 40 h gave long, large spontaneous contractions, which, in comparison to open, non-ligated segments of section V, showed alternating phases of maintained tone and slow relaxation over many hours (cf. experiments 24, 25, Table 4). In another experiment (experiment 17, Table 4), a segment of section IV, previously stored for 94 h, was ligated and distended with non-aerated RS-F_o solution (pH 7.09 ± 0.03). Periodic phases of activity with variation in tone (*k*) in this preparation were observed as with the in situ ligated segments of section V (experiments 24, 25; Table 4).

Interestingly, examination of the gut contents from ligated segments of section V after a 5 h storage period followed by 40 h perfusion indicated that a luminal pH between 6–8 had been sustained and that endosymbiotic diplomonad flagellates were still motile (experiments 24, 25; Table 4). Ligated (closed) gut preparations in these experiments showed no evidence of increased intraluminal pressures (swelling) during perfusion in RS-F_o.

Table 4 Experimental protocol and activity profiles for gut segments from *O. pullus* stored and perfused with RS-F_0. Numerals preceding *h* (in *parentheses*) indicate number of hours of duration (*k* constant, *L* ligated with in situ gut contents, *LR* ligated and distended with RS-F_0, * 2nd day of perfusion, ** 3rd day of perfusion, ↑ increase in tension, ↓ decrease in tension.

Experiment no	Gut segment	Storage @ 8–10 °C duration (h)	Time set in vitro @ 17 °C	Tension status	Perfusion duration (h)	Time of activity	Activity duration (h)	"Cold" response (hours since start of perfusion)
1	IL	56	2000	k	13	2100–2200	1	
						0130–0600*	5.5	
2	IL	70	1000	k	15	1600–2400	8	2,12
3	I	31	2300	k	23.5		None	15
4	I	30	2230	k	36	1300–1800*	5	24
5	II	69	1130	k	23		None	21
6	II	3	1700	↑(5 h), k(9 h), ↓(3 h), k(10 h)	27	1800–2400		16,19
						0000–0730*	13.5	
						1430–1500*	0.5	
						1800–2000*	2	
7	III	104	2300	k(3 h), ↑(5 h)	8.5	0000–0700*	7	
8	III	2	1630	k	18	1800–2100	3	
9	III	3.5	1530	k(32 h), ↓(5 h)	37	1700–1800	1	7,17,18,29
						1100–1500*	4	
						1800–2200*	4	
10	III	46	1015	k(4 h), ↑(10 h), k (4 h), ↓(4 h)	22	1500–2100	6	5,13
11	III	46	1015	k	22	1700–1900	2	3,13,22
						0000–0200*	2	
12	III	70	1000	k(9 h), ↑(4 h), k(2 h)	15	1500–1730	2.5	2,3,12
						2300–2400	1	
13	III	1	1500	↑(2 h) k(29 h)	31	1600–1800	2	25
						1500–1600*	1	
14	III	56	2300	k	24	2300–2400	1	16,24
						0100–0500*	4	
15	IV	45	1130	k	24		None	
16	IV	20	1100	k	24		None	
17	IVLR	94	1100	↑(8 h), k(3 h), ↓(10 h)	21	1800–2200	4	11
18	IV	21	1100	k	17		None	
19	IV	70	1200	↑	32	2000–2400		21
						0000–0600*	10	
20	IV	1	1500	↑(3 h), k(21 h), ↓(7 h)	31	1500–1800	3	19,25
21	IV	56	2245	↓(2 h), ↑(2 h), k(3 h), ↑(13 h), k(3.5 h)	23.5	0330–0430*	1	16
22	V	24	1100	↓(2 h), ↑(16 h), ↓(5 h)	23	1500–2400		
						0000–0400*	13	
23	V	45	1130	k	24	–	None	
24	VL	5	1530	↓(3 h), k(15.5 h)	18.5	1600–1700	1	7,18
25	VL	5	1530	↓(2 h), k(18 h), ↓(6 h), k(14 h)	40	0830–0930*	1	17
						1100–1700*	6	
						2100–2400*		
						0000–0400**	7	
26	V	33	2300	↓(2 h), k (7 h), ↑(6 h), ↓(8.5 h)	23.5		None	15

A total of 21 non-ligated preparations from *O. pullus* were perfused, of which 14 exhibited spontaneous activity. All five of the ligated preparations from *O. pullus* exhibited spontaneous activity. If activity is plotted against time of day for these preparations (Fig. 4), it can be seen that the bulk of activity falls between 1400 hours and 0600 hours. At the time of the study sunrise and sunset were approximately 0730 hours and 1700 hours, respectively. Therefore, most activity took place during the hours of darkness. We are unable to determine whether ligation influenced timing of activity. Only one of the five ligated preparations examined (experiment 25, Table 4) was active from 0800 hours to 1600 hours.

Gut morphology in *O. pullus* and *N. fucicola*

Transverse sections of the gut of these two fish species showed a marked difference in both the width of longi-

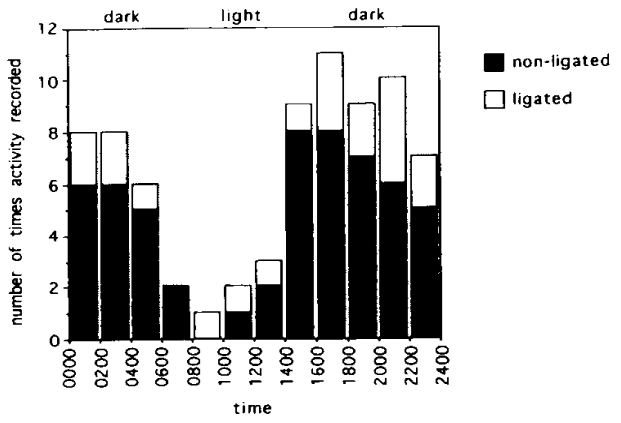

Fig. 4 Plot of activity against time of day for the 21 non-ligated and 5 ligated gut preparations from *O. pullus* perfused during the study. Of these preparations, 19 exhibited spontaneous activity. At the time of the study the times of sunrise and sunset were approximately 0730 and 1700 hours, respectively

tudinal and circular musculature and the overall diameter of the gut lumen (Figs. 5, 6; Table 5). The width of the circular muscle layer in both species was consistently greater than that of the longitudinal musculature (Table 5).

Discussion

One of the aims of this study was to develop a preservation/holding and perfusion regime to investigate physiological and biochemical aspects of digestion in herbivorous fishes. The first step was to generate realistic profiles of the plasma composition of study species. While the techniques employed ultimately gave consistent values for the ionic constituents measured in *O. pullus* (Table 1), there were two instances where elevated K^+ levels were recorded. These isolated instances could have resulted from unintentional damage of the musculature of the bulbous arteriosus, as suggested by Soivio and Oikari (1976). It is also possible that the elevated K^+ levels observed were the result of stress (Wells et al. 1986), although blood samples from *N. celidotus* taken using the same blood sampling technique gave consistent K^+ values.

The biochemical analyses of blood showed that the only major differences between the two study species were the levels of K^+, Na^+, glucose and osmolality. We found that using the K^+ and Na^+ values for the *O. pullus* perfusate ($RS\text{-}F_o$) in the formulation of the perfusate ($RS\text{-}F_n$) for *Notolabrus* and vice versa resulted in a dysfunction of gut activity. The importance of adopting the correct Na^+/K^+ balance can probably be explained in terms of the functioning of the K^+-dependent NaCl co-transporter system, as shown previously for flounder intestine (Palfrey and Rao 1983).

Factors such as oedema formation, loss of membrane selective permeability, and substrate depletion in isolated preparations are invariably the result of a failure to recognise the importance of utilising a balanced physiological in vitro environment. To maintain such an environment the normal biophysical environment must be re-created with respect to tonicity, osmolality and specific ion conductivity (Burton 1975; Rees 1989a). Unlike the empirically formulated "physiological" fish salines of previous studies (e.g. Burnstock 1958; Hudson 1968; Hirano et al. 1973), $RS\text{-}F_o$ and $RS\text{-}F_n$ were formulated using the activity coefficients of individual ionic species, not simply their total plasma concentrations, to give their "free" ionisable levels (Rees 1989a). For example, serum binding of Ca^{2+} and Mg^{2+} must be distinguished from the actual "free" ionised levels of these ions (Pederson 1973). Magnesium ions are important in a number of critical cellular reactions, and their extracellular presence is reported to stimulate mitochondrial respiratory activity and modulate the effects of rapid calcium influx (Sordahl and Silver 1975) and potassium eflux (Hearse et al. 1978).

Another important consideration in the formulation of RS-F was that it should maintain optimal levels of intermediate metabolites to preserve the physiological function of fish tissue and organs. Glucose and glycerol have been shown to meet the energy demands of isolated tissues and organs, even when they are not the preferred substrate for the organ (e.g. the heart), by the inclusion of physiological levels of insulin (Robiseck et al. 1985; Rees 1989a). Mammalian insulins such as that used in this study are very similar in structure to fish insulins (Christiansen and Klungsøyr 1987). Apart from their ability to be metabolised, glycerol and glucose also have free radical scavenging and membrane stabilising properties (Hess et al. 1983; Stewart et al. 1986), which are extremely important in maintaining the physiological viability of isolated tissues and organs. RS-F also contains the vitaminoid carnitine, which has multiple effects other than simply optimising oxidative metabolism (Whitmer 1987). Aspartate and glutamate were included to enhance oxidative metabolism by replenishing TCA cycle intermediates (Rosenkranz et al. 1986), thereby maintaining high energy phosphate levels. Glutamate is also involved in maintaining intracellular oxidation-reduction potentials (Safer 1975). By optimising the aspartate-malate and glycerol-phosphate shuttles, cells will maintain an optimal NAD/NADH balance and thereby sustain adenine nucleotide levels (Rosenkranz et al. 1986; Rees 1989a).

The natural physiological buffer system ($NaHCO_3/pCO_2$) was used for RS-F solutions in combination with the Good's zwitterionic buffer, BES [N,N-*bis*(2-hydroxyethyl)-2-aminosulfonic acid; Good et al. 1966]. BES has an ideal pK_a over the temperature range 10–37 °C, and has been shown to be non-toxic to cultured mammalian cells in long-term studies (Eagle 1971). Additionally, BES exhibits negligible binding of Ca^{2+} and Mg^{2+}, thus removing the potential hazard of precipitation of divalent cations over time that occurs when using conventional bicarbonate/phosphate or

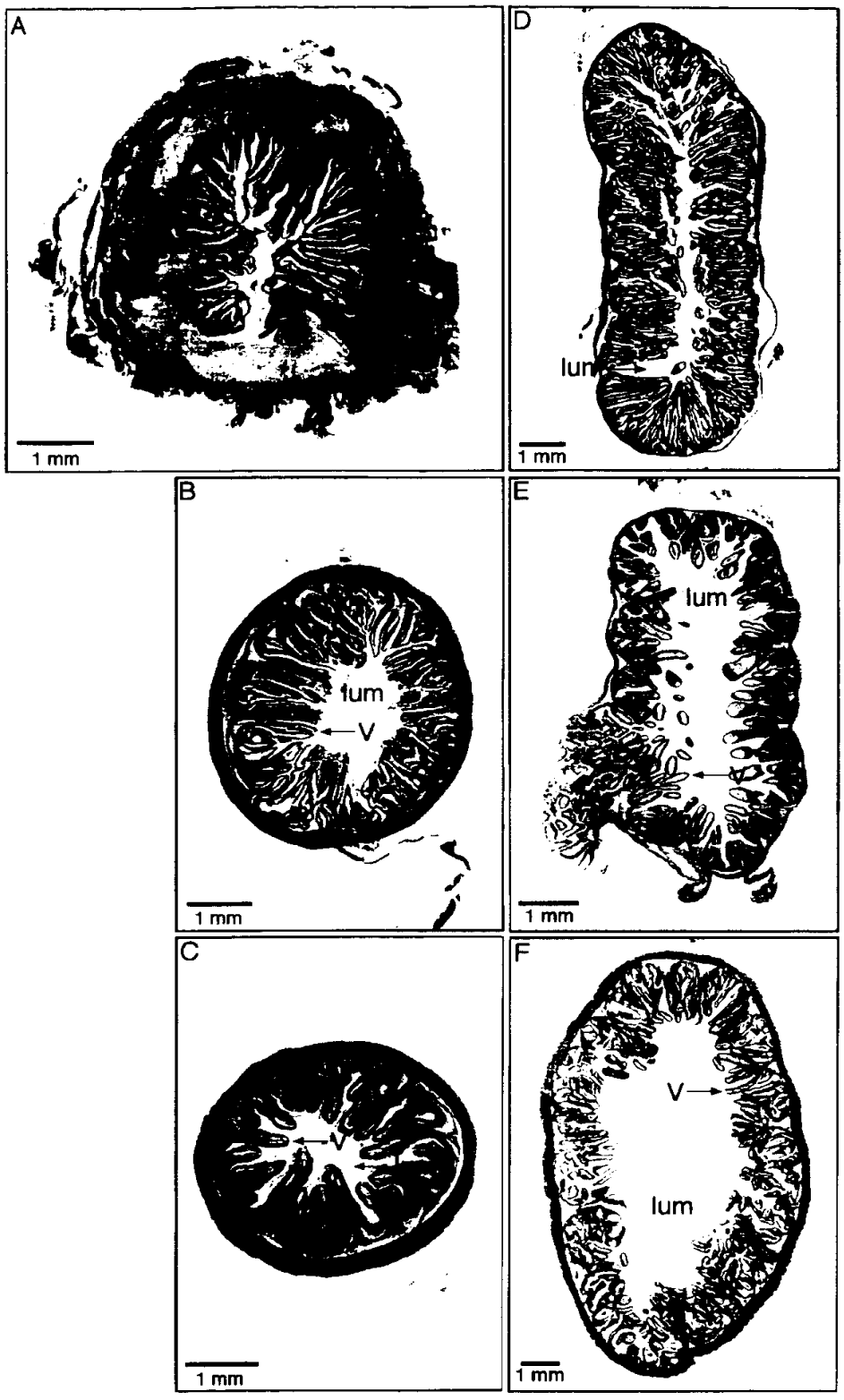

Fig. 5 A–F Transverse sections of gut segments from *N. fucicola* [190 mm standard length (SL)] and *O. pullus* (340 mm SL), showing overall gut morphology. **A–C** Sections I, II and III, respectively, from *N. fucicola*. **D–F** Sections I, III and V, respectively, from *O. pullus* (*lum* lumen, *v* villi)

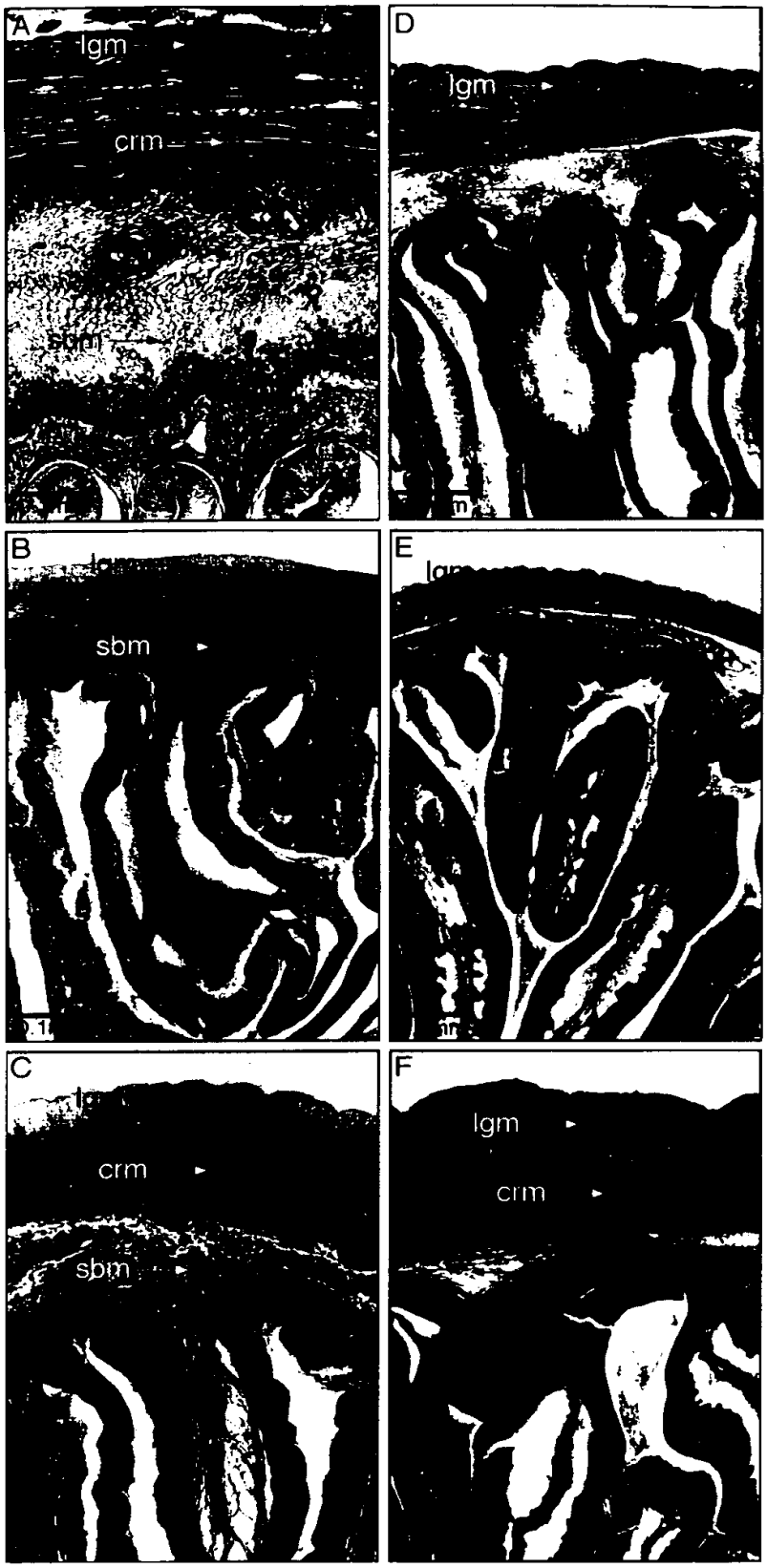

Fig. 6 A–F Transverse sections of gut segments from *N. fucicola* (190 mm SL) and *O. pullus* (340 mm SL), showing width of muscle layers. **A–C** Sections I, II and III, respectively, from *N. fucicola*. **D–F** Sections I, III and V from *O. pullus*. (*crm* circular muscle, *lgm* longitudinal muscle, *sbm* submucosa)

Table 5 Dimensions of circular and longitudinal muscle layers in transverse gut segments from *O. pullus* and *N. fucicola*. Values are mean μm ± SEM (n = 10). Note that gut of *O. pullus* was divided into five segments, gut of *N. fucicola* was divided into three. Standard length of *O. pullus* A was 240 mm, *O. pullus* B 340 mm, and *N. fucicola* 190 mm

Gut segment	Longitudinal muscle diameter			Circular muscle diameter		
	O. pullus A	*O. pullus* B	*N. fucicola*	*O. pullus* A	*O. pullus* B	*N. fucicola*
I	23.0 ± 9.7	21.9 ± 3.4	81.0 ± 6.4	61.3 ± 28.5	61.6 ± 9.3	186.3 ± 8.9
II	28.6 ± 7.6	33.8 ± 4.7	54.8 ± 5.2	57.8 ± 9.3	66.7 ± 7.9	129.1 ± 4.3
III	24.6 ± 8.6	18.4 ± 1.4	75.3 ± 6.1	29.4 ± 7.7	41.0 ± 2.7	186.3 ± 10.0
IV	27.8 ± 8.2	18.1 ± 1.9		31.9 ± 9.3	31.6 ± 2.9	
V	38.1 ± 10.5	87.5 ± 6.7		55.9 ± 4.8	122.3 ± 10.0	

double phosphate buffered solutions (Pederson 1973). Phosphate has traditionally been included as a buffer vehicle in conventional perfusion solutions for the past 80 years. This is despite the fact that inorganic phosphate ions have been shown to inhibit glycolysis and oxidative phosphorylation (Berman and Saunders 1955), creatine kinase activity (Hall and DeLuca 1986), and free radical scavenging enzymes (Stewart et al. 1986).

The activity we observed in gut segments from *O. pullus* and *N. fucicola* differed in both the magnitude and frequency of contractility. The differences in the magnitude of contractility are probably related to the dissimilarity between these species in both lumen size and the morphology of the circular and longitudinal musculature. However, this morphological dissimilarity does not explain the differences between the species in the frequency and timing of contractility. The continuous gut activity over periods of up to 43 h seen in the *Notolabrus* species was in complete contrast to the long intervals (1–21 h) of inactivity followed by periods of 0.5–4 h and 5.5–7 h of renewed activity observed in non-ligated and ligated segments, respectively, of *O. pullus* (Table 4). What factors might be responsible for these differences?

Intraluminal pressure is known to stimulate contractility in fish gut preparations (Fänge and Grove 1979). To assess the effect of intraluminal pressure on gut segments from *O. pullus*, we ligated gut segments distended with both in situ gut contents and RS-F_o. All five of the ligated preparations examined exhibited activity, with some suggestion that the amplitude of contractility was greater than in non-ligated preparations. However, we were unable to determine whether ligation influenced the timing of activity in *O. pullus* preparations, and none of the *Notolabrus* preparations were ligated when perfused.

Another possible explanation for the low level of activity observed in *O. pullus* preparations is the lack of short-chain fatty acids in RS-F_o. Short-chain fatty acids cause increased gut activity in the rat (Yajima 1985), and high levels of these metabolites occur in the posterior intestine of *O. pullus* under normal conditions (Clements et al. 1994). Two posterior gut segments ligated with in situ gut contents, and therefore presumably containing natural levels of short-chain fatty acids, did exhibit moderate levels of activity. However, in these experiments the effects of short-chain fatty acids are confounded with the effects of distension. Clearly, the effects of distension and short-chain fatty acids on gut activity in *O. pullus* require further work, but these factors alone cannot explain the differences in activity pattern between the *O. pullus* and *Notolabrus* preparations.

The activity pattern observed in isolated *O. pullus* gut segments is suggestive of a high frequency (ultradian) rhythm overlying a daily rhythm (Moore-Ede et al. 1982). Several of our preparations exhibited this rhythm even after storage at 8–10 °C for over 72 h. The persistence of daily rhythmicity in isolated tissue preparations has also been reported for rat hearts (Tharp and Folk 1965) and intestine segments from golden hamster (Bünning 1958). Feeding rhythmicity in fishes is thought to be controlled by a multi-oscillatory system, although the precise nature of this mechanism is unknown (Boujard and Leatherland 1992). Our results suggest that intestinal activity in *O. pullus* is partly controlled locally by neuron plexuses in the gut wall. Assessment of the daily rhythm in *O. pullus* gut preparations is complicated by variation in storage time, start time for each preparation, ligated versus non-ligated preparations, and our use of different parts of the gut (i.e. segments I–V).

An alternative hypothesis to a daily rhythm in activity is that the apparent rhythm exhibited by *O. pullus* segments was an artefact caused by setting up most preparations (i.e. commencing perfusion at 17 °C) during daylight hours. This hypothesis requires that there was a time-lag between the start of perfusion and the onset of muscular contractility in the *O. pullus* gut preparations, and that the cessation of activity reflects deterioration of the tissue rather than cessation of endogenous stimulation. However, the time-lag between the start of perfusion and onset of activity in the *O. pullus* preparations was quite variable (Table 4), and several preparations became active within an hour of perfusion. There are two additional reasons why this time-lag hypothesis is unlikely. First, two *O. pullus* preparations (experiments 6 and 9 in Table 4) became active at approximately 1800 hours for 2 days in succession after continuous per fusion. Second, the gut preparations from both of the *Not-*

olabrus species exhibited continuous, immediate activity under virtually identical experimental conditions, the only difference in experimental protocol being the formulation of the respective RS-F solutions. This difference between the study species suggests that the pattern of activity in the *O. pullus* gut preparations was unlikely to have been an artefact of the experimental conditions.

If the daily rhythm of contractility seen in *O. pullus* gut segments is not an artefact, what consequences would it have for the biology of the animal? The most obvious consequence is that a reduction or absence of peristaltic activity during the day will slow the movement of gut contents through the intestine. This will have the effect of increasing the residence time of food material, in this case macroalgae, in the gut. Slow passage of digesta is suggested as a requirement for fermentative digestion in vertebrates (Horn 1989), and presumably is a contributing factor to the high levels of short-chain fatty acids detected in the posterior gut of herbivorous odacids (Clements et al. 1994). These fishes seem to pack the gut during an intensive burst of feeding at dawn, and feed at low rates throughout the rest of the day (Choat and Clements 1993). Gut passage time has not been measured in herbivorous odacids. However, the low defaecation frequency characteristic of these fishes during daylight hours compared to other herbivorous species (K. D. Clements and D. Rees, personal observation), and their daily pattern of feeding, are suggestive of long gut transit times. The daily pattern of gut activity observed in *O. pullus* in this study may allow this fish to retain algal material in the gut throughout the day. During this time endosymbionts metabolise components of the diet, such as mannitol (Seeto et al. 1996), to short-chain fatty acids that are then absorbed and used for energy or lipid synthesis by the host fish (Clements et al. 1994). Undigested material is probably expelled at night, when the gut is active and the fish is inactive (Choat and Clements 1992).

In conclusion, the procedures used in this study enabled us to study the activity of isolated gut muscle preparations in vitro for extended periods of time. As a result, we observed major differences between the herbivorous *O. pullus* and the carnivorous *Notolabrus* species in both the amplitude and frequency of contractility. These differences in gut function appear to be related to the diet and mode of digestion of the study species: slow, fermentative digestion of macroalgae in *O. pullus*, and more rapid, endogenous digestion of invertebrates in the *Notolabrus* species.

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PHYSIOLOGICAL MEDIUM FOR PERFUSING, PRESERVING AND STORING ISOLATED CELL, TISSUE AND ORGAN SAMPLES

The present invention relates to the formulation of a physiological liquid medium having the basic, synergistic components to allow its universal application in preserving cellular and functional integrity *in vitro* of different cell, tissue and organ types isolated from different mammalian species.

Historically, the design of physiological perfusion solutions dates back to the thesis proposed by the French physiologist, Claude Bernard in the eighteen seventies who put forward his theory on the *milieu intériéur*, basically purporting that to maintain the *whole* (person) one should ensure that the surrounding extracellular environment should be balanced in all respects. Unfortunately, the misinterpretation or misconception of Bernard's *milieu intériéur* has led researchers to confuse the *extracellular* with the *intracellular* phases of cell function and largely to overlook the need to maintain the cell as a *whole* entity. The basic *salt* solutions currently used for *in vitro* or *isolated* organ/tissue studies derive from the simple formulation used by Sidney Ringer for the isolated perfused frog heart. Similar, empirically contrived, basic salt solutions have been utilised for isolated mammalian preparations. The conventional use of phosphate/bicarbonate buffered salines was instigated by Krebs and Henseleit (Z. Physiol. Chem. **210**: 33-66) for studies on isolated *homogenates* of mitochondria, ie. *intracellular* organelles, from pigeon liver. Later, Krebs, in his classical paper (Biochem. Biophys. Acta. **4: 249-269)** on the analysis of oxygen consumption in tissue slices from different organs in a variety of animal species, acknowledged that substrate depletion in isolated tissue/organ preparations *over time* was a consideration that had not been addressed in the composition of previous physiological solutions. As has already been demonstrated, a correct interpretation of Bernard's hypothesis necessitates that the *whole* cell should subtend metabolic homeostasis.

Traditionally, phosphate/bicarbonate buffers have been used for sixty years and are still currently used, with questionable validity, in perfusion/preservation solutions for mammalian and human tissues/organs. It is of interest to note that it has been known for 40 years that *inorganic* phosphate ions inhibit glycolysis and oxidative phosphorylation, creatine kinase and the enzymes involved in oxygen free radical scavenging, the latter being implicated in reperfusion injury and oedema formation in numerous organ systems. Maintenance of pH over time is further complicated by the instability of phosphate-buffered perfusion and preservation solutions caused by the precipitation of calcium phosphate and bicarbonate, accentuated by the change in their dissociation constants over the temperature range 4 - 37°C.

GB-A-2 270 614 describes an aqueous solution for the perfusion, storage and reperfusion of organs comprising calcium, potassium and magnesium chlorides, histidine, mannitol, lactiobionate, glutamate and glutathione.

GB-A-2 213 362 discloses a perfusate solution which comprises a lactobionate and a hydroxyethyl starch. The descriptive section of that document stresses the importance of including an adenosine triphosphate (ATP) precursor such as adenosine, in order to maintain the ATP level during reperfusion.

WO 98/04127 discloses a transplant solution comprising water, a buffer system and pyruvate.

The present invention provides an answer to the still-existing need to provide a solution to overcome the deleterious effects of inorganic phosphate ions on cell metabolism and associated cellular function while also serving to augment the natural physiological processes essential to preservation of isolated cell, tissue and organs from mammalian species, for instance during the transport of organs for transplant. Such organs deteriorate rapidly, and many useful organs cannot be used because too much time elapses between collection and delivery to an intended recipient. The solution according to the present invention extends the safe period.

The present invention provides a physiological medium which comprises an aqueous solution in sterile purified water of:

(i) a salt component comprising:

(a) from 100 to 150 mmoles /L of sodium ions,

(b) from 2.5 to 6.2 mmoles /L of potassium ions,

(c) from 0.1 (preferably from 0.15) to 2.5 mmoles /L of calcium ions,

(d) from 0.4 to 25 mmoles /L of magnesium ions, and

(e) from 96 to 126 mmoles /L of chloride ions;

(ii) a buffer component comprising

(f) from 21 to 27 mmoles /L of bicarbonate ions, and

(g) from 1 to 12 mmoles /L of TES, MOPS or BES;

(iii) a substrate component comprising:

(h) 2 to 11 mmoles /L of glucose

(i) 50 to 150 µmoles /L of glycerol and

(j) 7 to 15 µmoles /L of choline;

(iv) an amino acid component comprising:

(k) 5 to 400 µmoles /L of glutamate

(l) 5 to 200 µmoles /L of aspartate and

(m) 100 to 2000 µmoles /L of glutamine;

(v) a co-enzyme component comprising:

(n) 1 to 120 nmoles /L of thiamine cocarboxylase;

(vi) a vitaminoid component comprising:

(o) 40 to 70 µmoles /L of D- or DL- or L- carnitine;

(vii) a protein component comprising:

(p) 5 to 200 m I.U./L of porcine or human insulin; and

According to one embodiment, the salt component comprises

(c) from 1.0 to 2.5 mmoles /L of calcium ions, and

(d) from 0.4 to 2.4 mmoles /L of magnesium ions.

The present invention also provides a method for producing a physiological medium as described above which comprises adding in the following order: sodium chloride, potassium chloride, calcium chloride, magnesium chloride, the TES, MOPS, or BES, thiamine, carnitine, choline, glycerol, insulin, aspartate, glucose, glutamate, glutamine, and sodium bicarbonate to sterile purified water, with constant stirring, making up to the desired volume, filtering and storing in sterile sealed vessels.

It will be noted that the compositions according to the invention contain no animal derived serum protein, such as foetal bovine serum or bovine serum albumin, which have been banned by the FDA for such applications in single cell or human tissue/organ biotechnology.

Buffer components:

With regard to the components of the solutions according to the invention a natural physiological buffer system, namely $NaHCO_3$ / pCO_2, has been adopted for the solution in accordance with the invention, in combination with the zwitterionic Good's buffer, BES (Good *et al.* Biochemistry **5**: 467-477), which acts by virtue of its ideal pK_a over a temperature range of 10-37°C, to provide a stable pH, an essential requisite for cellular preservation. BES has been shown to be non-toxic to even cultured mammalian cells in long-term studies and exhibits negligible binding of Ca^{2+} or Mg^{2+}, so removing the potential hazard of precipitation of divalent ions which occurs when using conventional bicarbonate/phosphate or double phosphate buffer solutions. Indeed, 10x concentrates of solutions according to the invention have been experimentally shown to have a shelf-life (stored at 3-8°C) in excess of 14 months. As alternatives to the use of N,N-bis-(2-hydroxyethyl)-2-amino ethanesulfonic acid (BES), it is possible to use morpholinopropane sulfonic acid (MOPS) or N-tris-hydroxymethyl) methyl-2-amino ethanesulfonic acid (TES).

A major distinguishing feature of solution in accordance with the invention in comparison to conventional perfusion salines is the absence of inorganic phosphate which, for the past 80 years, has been used as the buffer vehicle in conventional perfusion solutions, even though inorganic phosphate radicals have been shown over the last 40 years to inhibit glycolysis and oxidative phosphorylation, creatine kinase activity, and free radical scavenging enzymes.

Inorganic phosphate has been reported to have an inhibitory effect on glycolysis by interfering with the co-operativity of Mg^{2+} with the rate limiting enzymes, hexokinase and phosphofructokinase, and on the solubility of creatine kinase. Numerous investigators, in an attempt to compensate for the inhibitory effect upon glycolysis and associated decline in physiological performance produced by phosphate buffered salines, have included pyruvate at non-physiological serum levels (i.e. 2.0-25.0 mmoles vs 0.2 mmoles) in their perfusate salines or to transplant solutions (see the above-mentioned WO 98/04127). Experiments conducted on the isolated, perfused/perifused rat liver have shown that leakage of lactate dehydrogenase (LDH) occurred after only 10 minutes with Krebs & Henseleit saline but not for 300 minutes with a solution in accordance with the invention (see Example 1). Under the latter conditions, the further addition of pyruvate would be contraindicated as pyruvate is known to cause inhibition of the H_4 and H_3M subunits of LDH in the liver and, incidentally, the heart. Again, it may be emphasised that for the preservation of isolated tissues/organs, all attempts should be made to retain metabolic homeostasis at the cellular level by maintaining `autoregulation' of enzymatic function.

The preferred salt component comprises:

> 135.32 mmoles /L of sodium ions, 5.00 mmoles /L of potassium ions, 1.25 mmoles /L of calcium ions, 0.45 mmoles /L of magnesium ions, as chloride salts, and 118.40 mmoles /L of chloride ions as sodium, potassium, calcium and magnesium salts.

The preferred buffer component comprises:

25.00 mmoles /L of bicarbonate ions as sodium salt and 5.0 mmoles /L of N,N-bis (2-hydroxy ethyl)-2- amino-ethanesulfonic acid (BES).

Substrate components

The solution according to the invention provides a number of essential substrates to retain metabolic homeostasis of the isolated organs/tissues. Glucose and glycerol have been shown to be satisfactory in meeting the energy demands of isolated tissues and organs, even when they are not the preferred substrate for the organ (e.g. the heart) by the inclusion of *physiological* levels of insulin. Apart from their ability to be metabolised, glycerol and glucose also have free radical scavenging and membrane stabilizing properties, which have been shown to be extremely important in maintaining the physiological viability of isolated tissues and organs. Preferably, the substrate component comprises 10 mmoles /L of D-glucose, 110 µmoles /L of glycerol and 10.0 µmoles /L of choline as the chloride salt.

Amino acid components:

Aspartate and glutamate have also been included in solutions according to the invention to enhance oxidative metabolism by replenishing TCA cycle intermediates, thereby maintaining high energy phosphate levels even during ischemic insult. Similarly, glutamate is involved in maintaining intracellular oxidation-reduction potentials. Essentially, it is suggested that, by optimising the aspartate-malate and glycerol phosphate shuttles, cells will maintain an optimal NAD/NADH balance and thereby sustain adenine nucleotide levels.

Preferably, the amino acid component comprises 300 µmoles /L of L-glutamate as sodium salt, 20 µmoles /L of L-aspartate as sodium salt and 400 µmoles /L of L-glutamine.

Co-enzyme components:

Thiamine cocarboxylase plays an essential role in the oxidation of α-keto acids, and is included in the compositions to prevent the accumulation of pyruvate and pyruvate aldehyde and thereby cell toxicity.

In the tricarboxylic acid cycle, TPP is a co-factor in the metabolism of α-ketoglutaric acid to form succinyl-coenzyme A, by oxidative decarboxylation, or to form glutamate, by reductive amination.

In essence, TPP is involved in numerous interrelated, biochemical pathways, especially those of the Pentose Phosphate and Glycolytic pathways.

The thiamine may, for instance, be employed as thiamine pyrophosphate or as thiamine diamide.

Preferably the coenzyme component comprises 40.0 nmoles /L of thiamine as thiamine pyrophosphate chloride.

Vitaminoid component

The vitaminoid, carnitine, has been reported to have multiple effects in improving cardiac function other than by simply optimising oxidative metabolism, such as, by promoting the utilization of alternative substrates and may additionally improve coronary blood flow. L-carnitine is preferred to the D- or DL-isomers, because it causes no inhibition of acetyl co-enzyme A / free fatty acid metabolism. Preferably the vitaminoid component comprises 50.0 µmoles /L of [-]-β-hydroxy-γ-trimethylamino-butyrate hydrochloride (L-carnitine). In this invention the inclusion of the L-isomer of carnitine in the formulation of the solution was intended to optimise the transport of long chain fatty acids from the cytosol into the mitochondrial matrix to the site of β-oxidation and thereby to buffer the intramitochondrial acetyl CoA/CoA ratio by stimulating the synthesis of acetyl carnitine from carnitine acetyltransferase. This reduction in the ratio of acetyl CoA/CoA will result in an efflux of acetyl carnitine from the mitochondria with an associated stimulation of pyruvate dehydrogenase and reversal of fatty acid inhibition of glucose oxidation. Ultimately, the optimisation of free fatty acid utilisation as an energy source is essential for all types of cells but this must be done with preservation of carbohydrate (glucose) utilisation by optimised functioning of the enzymes involved in glycolysis, eg. hexokinase, glucokinase, phosphofructokinase.

D- or DL- isomers of carnitine are less effective in fulfilling this function.

Insulin components:

The use of human recombinant insulin (expressed in E. Coli) not only precludes the risk of antigenic or viral contamination in recipient cells/tissues/organs, as may be the case with insulin derived from other mammalian or animal species, but leads to a better fit being achieved of insulin molecules to human insulin receptor structure, ie. receptor specificity will be optimised to retain the many associated functions of insulin in cellular processes.

Preferably the protein component comprises 28.0 m. I.U./L of recombinant human insulin (expressed in E.coli).

Few of the disclosed formulations for perfusate and preservation solutions over the last 60 years having included insulin, and those that have, made use of insulin at unnatural levels, e.g. 10- 50 x 10^6 mIU/L (i.e. about a million times more concentrated than in the compositions according to the invention). The reason for this relates to the fact that only a small amount of the insulin exists as single molecules in such concentrations. The rest of the insulin exists as large aggregates of insulin molecules which are ineffective in action, i.e. individual molecules of insulin are needed to stimulate insulin receptors on cell membranes.

Essentially, the biochemical effects of insulin do not simply relate to its ability to regulate carbohydrate metabolism and facilitated transport of circulating glucose into cells but also (i) the enhancement of intracellular glucokinase activity and amino-acid incorporation into proteins, (ii) stimulation of DNA translation, (iii) increased lipid synthesis, and (iv) stimulation of sodium, potassium and inorganic phosphate into cells.

The invention acknowledges that in the absence of species-specific insulin there will be profound changes in the entire balance of cellular metabolism, eg. increased gluconeogenesis from protein, increased lipolysis and ketogenesis. The net result will be a total disruption in metabolic homeostasis and cellular function.

The use of human recombinant insulin in the compositions according to the invention, in preference to animal serum-derived insulin, relates not only to meeting FDA regulations but to the fact that a better fit will be achieved of the insulin to the human insulin receptors, i.e. receptor specificity will be optimised.

Therefore, normal serum levels of insulin have been utilised in the compositions according to the invention and this could only be achieved by acidifying the insulin to prevent aggregates of insulin forming so allowing individual molecular species of insulin to exist in solution.

Using alkali or neutral pH solutions do not achieve this molecular dispersion of insulin molecules.

Antibiotic components:

Preferably, an antibiotic component is included to ensure that, should there be any accidental contamination by micro-organisms during transport, any multiplication of such micro-organisms can be prevented. Preferably, the antibiotic component comprises 10 to 150 mg/L, more preferably 100 mg/L, of D-[-]-theo-2-dichloroacetamide-I-(p-nitrophenyl)-1,3-propane acid (chloramphenicol). Other antibiotics may be used, but care must be taken to ensure that the particular antibiotic employed does not interfere with the tissues or organs being stored or transported.

Ionic:

The concentrations of ionic species in solutions according to the invention acknowledges the activity coefficients of each ionic species and not simply their total serum concentrations. For instance, serum binding of Ca^{2+} and Mg^{2+} must be distinguished from the actual free, ionised levels of these ions. Magnesium ions are important in a number of critical cellular reactions and their extracellular presence is reported to stimulate mitochondrial respiratory activity and modulate the effects of rapid calcium influx and potassium efflux. Equally, an adequate concentration of calcium ions must be present in the preservation solution to avoid the calcium paradox, observed upon subsequent exposure of the donor organ to total serum calcium levels upon reperfusion and transplantation. Additionally, the ionic conductivity of solutions

according to the invention is comparable to that of human serum, namely 12.6 mS cm^{-1} and as such maintains the ionised status of the cell membrane and activities of enzymic moieties. Of significant concern in the majority of preservation solutions are the levels of potassium and sodium, in that the potassium ion concentration is some twenty-five times higher and sodium five to fifteen times lower than their serum levels.

Osmolarity:

A solution according to the invention is isosmotic to human serum (ca. 290 mOsmoles/L) and does not appear to necessitate the inclusion of plasma expanders, as demonstrated by the fact that only minor changes (ca. 8%) in hydration occur during long term (i.e. 4-52 h) hypothermic perfusion of the isolated rat heart and visceral nerve-muscle preparations. This may be explained by the fact that the cell membrane lies in continuity with a 99% gel interstitial phase so providing natural colloidal buffering to excess Donnan ionic equilibrium exchange across the cell membrane. The majority of the osmotic pressure is provided by Na^+ and its accompanying anions and only a small component (ca. 0.5%) can be attributed to plasma proteins and thereby has not justified the inclusion of oncotic agents in solutions according to the invention. The practicality of including oncotic agents, as in other commercially available perfusate/preservation solutions, is further compromised by their affinity for Ca^{2+} and Mg^{2+}, necessitating prior dialysis in fresh solution so as not to disturb the cationic composition of the perfusate. The labile nature of polypeptide expanders also makes them impractical through their predisposition to mechanical denaturation as occurs in their preparation and circulation through perfusion apparatus. Unfortunately, while these colloidal expanders are essentially non-toxic, their use in preservation fluids is contraindicated in terms of, for example, (**1**) raised viscosity increases the thickness of the 'unstirred' layer around cells so hindering diffusion of metabolites, (**2**) alteration of the surface membrane bioelectric potential so disrupting cellular metabolism and receptor activities, (**3**) antigenicity of proteinaceous expanders, (**4**) agglutination and haemolysis of RBC's and (**5**) blockage of microvasculature and ischemia.

Amongst potential uses for the solutions according to the invention are as a flushing/holding/preserving/transporting medium for Human bone marrow cells at 4°C (see Example 6) and Mammalian embryos (among others) for 12-48 hours at 20 to 37°C (see

Example 4). In agriculture this could be used for embryos of such species as sheep, goats, deer, cattle, pigs and horses.

Another potential use is as a non-frozen solution for use as a medium for storing semen for 24-48 hours at 20 to 25°C. The techniques heretofore used for this purpose are restricted solely to freezing the sperm with highly variable success. The solution is particularly useful for porcine semen since porcine semen cannot be frozen as compared to bovine semen.

In vitro applications in accommodating a variety of animal organ/tissue preparations used in physiological and pharmacological bioassay techniques for scientific experimentation at student to research levels of exploration, e.g.

a) perfused (cannulated)/perifused mouse, rat, guinea pig heart/heart-lung, liver, kidney preparations

b) perifused visceral muscle preparations, e.g. blood vessels, G.I. tract, reproductive tract biopsies

c) perifused mammalian skeletal muscle biopsies, e.g. mouse, rat, rabbit, human

d) perifused tissue slices, e.g. liver, brain

The preparation of solutions according to the invention is sensitive to the method by which stock solutions are prepared and stored, and to the order in which they are incorporated. The following Example is given as an instance of the preferred method of combining the various ingredients into the compositions.

Example 1

*Preparation of **RS-I** solution*

In the following, thiamine pyrophosphate (cocarboxylase), Sigma C4655 was prepared as a 0.4 mg/mL stock solution in MilliQ (endotoxin-free) purified water, and stored frozen in dark glass vials. Choline chloride (Sigma C7527) was prepared as a 17.5 mg/mL stock solution in MilliQ endotoxin-free purified water and stored frozen in glass vials. Human recombinant insulin (Sigma I0259) was prepared as a 0.5 I.U./mL stock solution in endotoxin-free MilliQ purified water acidified to pH 2.4 with 0.1N hydrochloric acid and stored frozen in glass vials.

In the following preparations, endotoxin-free MilliQ purified water was used throughout, both in the initial stirring, and in the final dilution.

For the preparation, a stainless steel container was filled with 8 litres of MilliQ and, the following ingredients were weighed out and added while constantly stirring, in the following order: 642.96 grams of sodium chloride (CFK0484), 37.28 grams of potassium chloride (BDH10198), 18.38 grams of calcium chloride dihydrate (BDS10117), 9.14 grams of magnesium chloride hexahydrate (BDH101494) and 106.61 grams of BES free acid (Sigma B6266), 1.84 milligrams of thiamine pyrophosphate (Sigma C9655) (using 4.6 mL of the stock solution), 0.9899 grams of L-carnitine (Sigma C0238), 0.1397 grams of choline chloride (Sigma 7527) in the form of 8 ml of the stock solution, 1.013 grams of glycerol (Sigma G2025), 2.8 I.U. of human recombinant insulin (5 ml of the stock solution), 0.310 grams of L-aspartate sodium salt (Sigma A6683), 180.2 grams of anhydrous D-glucose (Sigma G7021), 5.07 grams of L-glutamate sodium salt (Sigma G5889) and 5.84 grams of L-glutamine (Sigma G5763). The whole was stirred until completely dissolved and then the final volume of 10 litres was produced by adding further MilliQ purified water.

The solution was filtered through a sterile filter (0.2 μm Sartobran PH) into 100mL sterile sealed glass bottles.

This solution is a 10x concentrate of the solution intended for use. When needed, it can be diluted with the appropriate quantity of MilliQ.

For use as a perfusion and preservation solution, 100ml of the concentrate may be diluted with 900 mL of double deionised or endotoxin-free MilliQ purified water to 1 litre with the addition of 2.1 g of endotoxin-free sodium bicarbonate (Sigma S4019) and stored at 8-10°C prior to use. Sodium bicarbonate is not added to the concentrates before they are stored, since extended storage of the concentrate containing bicarbonate ions may cause precipitations of calcium carbonate.

For use as a perfusion and preservation solution, each litre of solution may contain 100 mg/L of chloramphenicol (Sigma C3175) to prevent the risk of bacterial contamination as may occur during extended periods of *in vitro* experimentation under exposed, atmospheric conditions.

The following factors are very important in the preparation of compositions according to the invention. The most critical factors are:

1. The method of assembly of the solutions according to the invention and, specifically:

2. Use of endotoxin-free MilliQ water to make up all stock solutions and the 10x concentrate bottles of manufactured solutions according to the invention.

3. The method of preparing sterile stock solutions according to the invention and concentrates not involving autoclaving or irradiation - e.g. irradiation to achieve sterility resulted in degradation of glutamine and probably TPP.

4. The use of glass bottles for all storage of stock and 10x concentrates of solutions according to the invention.

5. Preparation of solubilised insulin by acidification at pH 2.4 plus storing insulin stock solutions frozen.

6. Preparation of thiamine pyrophosphate plus TPP stock solutions stored frozen under dark conditions (see reason below).

7. Preparation of choline-chloride plus stock solutions stored frozen.

8. Use of magnesium chloride hexahydrate (i.e. 6 H_20). This is because if the dehydrate salt is used then as it adsorbs water the weight used to calculate the precise magnesium ion content will be in error - this is a common reason for wrongly made up Krebs solutions in terms of correct Mg-ion and Ca-ion levels.

It is not possible to omit any of the preferred components when formulating solutions according to the invention. All of these components work in synergy to produce the overall balanced physiological effect. This is why the use of human insulin and L-carnitine will only add to the overall attainment of viability of this versatile mammalian liquid medium.

Manufacture:

1. Stock solutions: Various stock concentrations of solutions according to the invention namely, 1x, 10x and 20x for long-term storage have been prepared and trialed successfully, but the preferred stock concentrates are 10x concentrates using endotoxin-free milliQ water and sterile filtered into sealed 100mL bottles for storage under dark conditions at 3-8°C. Stock solutions are reconstituted for use as 1x concentrate solutions by the addition of 100mL of 10x concentrates of stock solutions to 900mL of double deionised or endotoxin-free MilliQ purified water with the addition of 2.1g of sodium bicarbonate to give a final pH of 7.22 ± 0.04 at 20°C. Sterile stock 10x concentrations of solutions according to the invention have a pH of 4.6 ± 0.2 and have been shown to be retained as such for periods of up to five years. The recommended manufactured shelf-life of 10x stock concentrates of solutions according to the invention is 14 months when stored at 3-8°C.

2. Cocarboxylase: Stock solutions of thiamine pyrophosphate chloride (cocarboxylase) are prepared at 18.4 g/mL using endotoxin-free milliQ purified sterile filtered into dark sealed vials to prevent the photon degradation of thiamine pyrophosphate and stored frozen prior to the assemblage of 10x stock concentrates of solutions according to the invention.

3. Insulin: Human recombinant insulin is prepared as acidified (pH 2.4) stock concentrated solutions at 0.5 m I.U/mL using endotoxin-free milliQ purified water and sterile filtered into sealed vials and stored frozen prior to the assemblage of stock concentrates of solutions according to the invention.

4. Choline: Stock solutions of choline chloride are prepared at 17.45 mg/mL using endotoxin-free milliQ purified water and stored frozen in sealed vials prior to the assemblage of stock concentrates of solutions according to the invention.

5. Chloramphenicol is not an essential component of solutions according to the invention but is preferably added, either for storage, or after the storage vials have been opened, to ensure sterility during extended exposure of the solutions to the atmosphere, e.g. during perfusion or perifusion, or non-perfused preservation procedures.

The following further Examples show the use of solutions in accordance with the invention.

The accompanying drawings are graphs which are explained in the Examples, as follows:

Figure 1 is a graph explained in Example 2;
Figure 2 is a graph explained in Example 3; and
Figures 3, 4 and 5 are graphs explained in Example 6.

Example 2

Preservation of physiological and pharmacological functions

A physiological medium according to the invention, prepared in accordance with Example 1, and hereinafter called **RS-I** solution, has been successfully used as a perfusion and preservation medium in physiological and pharmacological experiments, using a variety of different tissues and organs, isolated from a variety of mammalian species, including human biopsies.

The design of the formulation is based upon the chemical and physical composition of human serum which therein relates to its 'universal' application and the results achieved to date (see Table 1).

The use of a non-phosphate buffer system to maintain stable pH values of isolated embryos (see Example 5) and human cells (see Example 6), and tissues/organs during storage at 3 - 10°C or perfusion at 20 - 37 °C (Table 1) supports the contradictory use of conventional phosphate buffered media (Table 2).

This point has been validated in experiments designed to compare the adverse effects of phosphate buffered media (e.g. Krebs & Henseliet; Dulbecco) versus non-phosphate buffered RS-I solution (see Table 3). The use of phosphate buffered solutions is contraindicated in the storage of liver transplants based on the results achieved in isolated rat liver biopsies, where a significant loss of lactate dehydrogenase (LDH) was observed after only 10 minutes of perfusion with Krebs & Henseleit perfusate (Fig. 1). The addition of pyruvate to the perfusate (as proposed by others, e.g see WO 98/04127) to compensate for the inhibition of glycolysis by phosphate ions, is contraindicated, as pyruvate is known to cause inhibition of the H_4 and H_3M subunits of LDH in the liver and would simply accentuate the observed deterioration.

Table 1 Functional viability of RS-I maintained mammalian tissue/organ preparations

Species	Tissue/Organ	Max" Days Preparations	Preservation Conditions Stored *in vitro* °C	Exp. °C
rat	jejunum	9.0	8-12	35
"	"	1.5	-	35
"	ileum	8.0	8-12	35
"	"	1.3	-	35
"	colon	5.0	-	20-35
"	uterus	3.0	-	35
"	"	10.0	8-12	35
"	detrusor muscle	2.0	-	20-35
"	diaphragm muscle	0.6	-	35-37
"	" "	2.0	-	20-35
"	soleus muscle	1.1	-	20-35
"	heart	0.8	-	35-37
"	"	2.1	-	20-25
"	heart-lung	1.2	-	20-35
"	RBC's	4.0	No haemolysis at 4°C	
"	kidney	1.0	-	20-35
"	liver	0.3	-	35
rabbit	intestine	5.0	8-12	37
"	"	2.0	-	20-37
"	uterus	7.0	8-12	37
"	superior cervical ganglion	2.0	8-12	37
"	" "	0.8	-	37
"	RBC's	3.0	No haemolysis at 4°C	
guinea pig	ileum	7.0	8-12	37
"	" detrusor muscle	4.0	8-12	37
"	" "	1.0	-	20-37
"	" heart	0.4	-	20-37
mouse	soleus	0.9	-	20-35
"	diaphragm	1.5	-	20-35
"	intercostal } mepp	0.9	-	20-35
"	diaphragm } discharge	1.5	-	20-35
human	intercostal analysis	1.3	-	37

Table 2 **Comparative performances of isolated tissue/organ in RS-I solution versus conventional phosphate buffered solutions**

Species	Preparation Type	Experiment	Solution	°C.	Number	Time (hrs)	Survival %	Function %	RS-I Comparative % Performance of: Survival	Function
RAT	Vastus Lateralis	malignant hyperthermia clinical trials	**RS-I**	35	12	8	**80**	**90**	**+800%**	**+150%**
			Krebs	35	12	8	10	60		
RAT	Langendorff Heart	effect of RS-I and RS-I with phosphate buffer	**RS-I**	35	6	4	**100**	83	**+500%**	**+400%**
				35	16	4	20	20		
		effect of *mild* hypothermia and *conventional* hypothermia	**RS-I**	15	5	24	**90**	**65**	**+900%**	**+550%**
			RS-I	8	6	16	10	12		
RAT	Liver	retention of LDH with perfusion.	**RS-I**	35	5	5	**60**	**100**		
			Krebs	35	5	5	0	0	(Krebs : **total failure**)	
GOAT	Embryos	incubated in (a)	**RS-I**	37	48	>12	**75**	**100**		
		vs. (b)	Dulbecco's	37	55	>12	13	100	cf. (b) **+570%**	**N/A**
		vs (c)	Whittingham's	37	58	>12	22	100	cf. (c) **+340%**	**N/A**

Attention is drawn to Fig. 1, a comparison of the lactate dehydrogenase (LDH) activity measured in perfusates from the isolated rat liver during perfusion / perifusion with RS-I solution (●— ●) and Krebs-Henseleit saline (o---- o) in a Res-Del® perfusion bath. An analysis of the time course of leakage of LDH using a repeated measure Anova programme indicates a highly significant ($p < 0.001$) leakage of this intracellular enzyme from isolated rat liver preparations (n=5) maintained in K & H saline verses RS-I solution. Note that the leakage of Lactate Dehydrogenase (LDH) from preparations maintained in Krebs-Henseleit saline occurred within the first 10 minutes while LDH leakage in RS-I perfusates was not significant until 90 minutes and only showed a definite leakage after 240 minutes.

Each bar indicates standard error of the mean (SEM).

Example 3

As a diagnostic solution for drug bioassay evaluation

The standardised and stable composition of RS-I solution in terms of storage over time, a characteristic not found with conventional phosphate buffered solutions, ensures that the isolated preparations in each drug trial exhibit normal physiological responses in comparison to the decremental responsiveness observed in phosphate buffered solutions (e.g. Krebs & Henseleit, Tyrode's, Hank's salines).

In experiments designed to test the validity of using RS-I solution in preference to conventional Krebs & Henseleit saline on isolated rat diaphragm preparations, it was observed that after 1 hour of perfusion there was a 18.6% decrease in the neurally evoked twitch response compared to a 2.9% decrease in RS-I perfused preparations (Table 3).

This observation was further validated in experiments to demonstrate the potentiating effect of betamethasone (*Betnesol*® ;Glaxo Ltd, NZ), on neurally evoked twitch responses in this preparation (Parr et al. British J. Anaesthesia, **67,** 447-451; Robinson et al. Anesth. Analg. **74,** 762-765) where it was imperative that the preparations should not exhibit any decrement in

tetanic fade under 'control' conditions during experimental time periods, in order to validate the hypothesis that betamethasone had a potentiating effect of neurotransmitter release. Such was not found to be the case using Krebs and Henseliet saline even during the first 30 minutes of the experimental time period (see Table 3 and Fig. 2) .

In another series of experiements, the classical guinea pig ileum preparation was trialed in RS-I solution and found to continue functioning and exhibiting normal pharmacological responses for up to 7 days. Such preparations have been alternatively stored in RS-I solution at 8 - 10 °C and then successfully used in drug trials so saving the number of animals having to be sacrificed.

It is therefore believed that the validity of any drug bioassay responses recorded using a phosphate buffered solution as a diagnostic perfusate is seriously compromised by the deleterious effects previously reported to occur (Table 2).

Table 3. Twitch response data recorded from rat diaphragm preparations perfused with RS-I and Krebs & Henseleit solutions at 35 °C

Time (min)	RS-I solution		Krebs & Henseleit saline	
	n	% Response‡ [mean ± SEM]	*n*	% Response‡ [mean ± SEM]
30	8	0.2 ± 1.7	10	11.5 ± 2.7
60	8	**2.9 ± 1.8**	9	**18.6 ± 2.7**
90	8	7.0 ± 1.3	8	27.2 ± 4.3
120	8	10.2 ± 2.6	9	31.2 ± 3.8
150	8	14.9 ± 3.5	10	36.1 ± 3.7
180	7	18.8 ± 4.0	9	38.0 ± 3.6

‡Responses are expressed as the percentage twitch contraction at 30 minute intervals compared to the initial twitch contraction at time zero.

Example 4

As a cardioplegic solution in Heart By-pass and Transplant Procedures

The advent of heart bypass and transplant surgery has accelerated the search for improved cardioplegic vehicles, because increasing the cardioplegic holding time will allow more involved cardiac surgery to be performed, without the present 45 minute time constraints.

Equally it would enlarge the donor pool area in terms of time and the current geographical restraints, and facilitate more efficient use of donor organs by overcoming tissue crossmatching in the recipient.

A cardioplegic solution may be used to serve two main purposes. Firstly in the situation of *in vivo* surgery, such as coronary bypass and valve replacement surgery, where the heart is stopped for a relatively short period (less than 45 minutes), with a combination of hypothermia (mild or extreme) and one of a number of cardioplegic solutions.

Secondly, a situation may exist for the use of a cardioplegic solution in the case of transplant surgery, whereby the heart is excised from the donor and transported in a cardioplegic preservation solution, RS-C, e.g. RS-I with 25.0 mmmoles/L of magnesium sulphate, to the recipient so as to preserve the metabolic status of the tissue.

The principal aims of a cardioplegic solution as summarised by Buckberg (J. Thoracic Cardiovasc. Surg. 77, 803-815) are to:

* protect the myocardium from the deleterious effects of ischaemia.
* reduce the energy requirements of the muscle, but provide an environment where energy production continues.
* arrest the heart safely.

A cardioplegic solution must fulfil these aims as well as prevent the negative effects of acidosis and oedema and stabilise the membrane to prevent unnecessary loss of intracellular ions.

In this study a horizontally aligned version of the *Langendorff* heart preparation was isolated in a Res-Del® 589 Perfusion Bath System and used to assess the viability of cardioplegic RS-C. The modified *Langendorff* preparation used, enabled simultaneous measurements of the heart rate and isovolumetric changes in the preparation, via an on-line pressure transducer. Coronary flow rates and an analysis of the electrocardiographic activity were also measured.

The cardioplegic techniques employed was either one of *continuous* infusion of the cardioplegic solution in the retrograde direction or , as conventionally practiced in heart by-pass surgery, a 'single' opposed bolus injection of the cardioplegic solution. The supposition was made that a *continuous* infusion would preclude the development of ischaemia or hypoxia during cardioplegic episodes. The results achieved in Tables 4 - 6 indicated that a 100% preservation of functional activity in the arrested hearts had been achieved for periods of up to six hours over a temperature range of 20 - 35 °C.

Interestingly, the percentage recovery did not appear to depend on coronary flow rates which were negative in experiments conducted over 3 - 6 hours.

Table 4. Effect of perfused RS-C solution on the Res-Del® *Langendorff* rat preparation

HEART Nos.	CP - PERIOD (hr)	CFR % CHANGE DURING CP	% RECOVERY WD @ 5 min	% RECOVERY WD @ 40 min	CFR % CHANGE @ 40 min
6	1	+3	+83	+22	-12
6	3	-40	+68	-7	-46
6	6	-49	-20	+5	-56

Table 5. Average times to cessation and recovery of **'cardiolplegic'** Res-Del® *Langendorff* rat heart preparations

HEART Nos.	1 hour CP-PERIOD		3 hour CP- PERIOD		6 hour CP- PERIOD	
	STOP	START	STOP	START	STOP	START
6	34	142	25	93	44	91

Table 6. **Effect of 'Single' bolus RS-C solutions on the Res-Del® *Working* rat heart preparation**

HEART Nos.	CP-PERIOD	% RECOVERY WD @ 10 min	% RECOVERY WD @ 40 min	CFR % CHANGE @ 40 min
4	1	+1	+18	-56
4	3	+33	+21	-40

Example 5

As a 'flush' and 'hold' solution in Animal Embryo Transplant Procedures

In independent trials, RS-I solution was trialed as a 'flush' , 'hold' and incubation medium for Mammalian embryos (among others) against commercially available phosphate buffered media over 12 -18 hours.

In these experiments goat embryo were incubated in Dulbecco's, Whittingham's and RS-I media at 38°C for 12-18 hours to assess survival rate.

The results indicated that RS-I solution offered superior preservation (75%) in comparison to Dulbecco's (24%) and Whittingham's (38%) for medium to long-term incubation of embryos (Table 7).

In another independent study conducted on cattle embryos, the selection of the Good's buffer, BES, was assessed in comparison to the Good's buffers, HEPES and MOPS and conventional phosphate buffering solution, PBI.

These results indicate that only in RS-I/ BES buffered solution did the embryos show any improvement in development (Table 8) even though compromised by initial incubation in PBI solution and an anticipated inhibition of their metabolic status (ref. Table . 2).

Table 7. Comparative study on RS-I solution as a medium for goat embryo preservation

TRIAL MEDIUM	EMBRYO NUMBERS *		
	'SURVIVED'	'DEGENERATE'	TOTALS
Dulbecco's PBS + 10% goat serum	13 **(24%)**	42 **(76%)**	55
Whittingham 's + 10% goat serum	22 **(38%)**	36 **(62%)**	58
RS-I solution	36 **(75%)**	12 **(25%)**	48

* Embryos were incubated 12-18 hours at 38 °C with 100% humidity

Table 8. Assessment of RS-I [BES], HEPES-HOLD, MOPS-HOLD and PBI-HOLD solutions as a culture medium for cattle embryo

Medium	N	% Poorer	% Same	% Improved
HEPES / HOLD	12	17	75	8
MOPS / HOLD	13	8	92	0
BES / RS-I HOLD	**15**	**40**	**40**	**20**
PBI-HOLD	15	27	73	0

The RS-I results in these trials were compromised because ALL cattle embryos were 'flushed' and 'held' in BSA-enriched phosphate solution **(PBI)** for up to **6 hours** prior to experimentation in the different types of Good's buffered media.

The additional use of RS-I in such applications in embryo preservation during `hold phases' resides in the fact that it has proved to be equally effective under *mild* hypothermic conditions in terms of cell, tissue and organ preservation.

Example 6

As a 'holding' Media for Human Normal Bone Marrow

In independent trials conducted at Life Technologies Inc. N.Y, USA, RS-I solution was assessed against conventional serum supplemented or serum free media formulations as a holding medium for the temporary storage of freshly harvested human bone marrow cells.

The experimental procedures involved mixing one mL of bone marrow with one mL of the following test media:

- IMDM + 20% FBS
- StemPro™-34 without recombinant growth factors
- RS-I/Res-Del media lot 3106
- RS-I/Res-Del media lot 7002

The samples of media and marrow were then placed at 4°C. Aliquots of the marrow were stained with antibodies for flow cytometric analysis.

Additional aliquots of the bone marrow were seeded into StemPro™-34 supplemented with the human recombinant growth factors: Stem Cell Factor (100ng/mL), IL-3 (50ng/mL) and GM-CSF (25ng/mL). The cells were incubated for six days at 37°C in a humidified atmosphere of 5% CO_2 and air.

After storage at 4°C for 24 and 48 hours, aliquots of the bone marrow in the indicated media were taken for cell counts and viability as described above. Aliquots were also stained for flow cytometric analysis and *ex vivo* cell expansion as described.

During the time course of this study, cell viability as determined by Trypan Blue dye exclusion, remained essentially 100% for the bone marrow cells, irrespective of the media formulation. During storage at 4°C there was a slight but not significant increase in cell number in all of the formulations tested (see Fig. 3).

The proliferative potential of the progenitor cells in the bone marrow by culturing aliquots of the marrow in StemPro™-34 supplemented with a combination of human recombinant growth factors demonstrated to promote cell expansion.

Aliquots of the bone marrow cells stored in the various media formulation tested were cultured in StemPro-34 supplemented with the human recombinant growth factors SCF (100ng/mL),

IL-3 (50ng/mL) and GM-CSF (25ng/mL). The cells were grown for 6 days and then cells counts determined using a Coulter Counter.

All of the media formulations tested showed a similar decline in the proliferation of the progenitors cells after 24 and 48 hours storage at (Fig. 4).

It was concluded that RS-I solution could be used as an alternative to phosphate buffered media to circumvent the published deleterious effects on isolated mammalian cells, tissues and organs (See Table 2).

CLAIMS

1. A physiological medium which comprises an aqueous solution in sterile purified water of:

(i) a salt component comprising:
- (a) from 100 to 150 mmoles /L of sodium ions,
- (b) from 2.5 to 6.2 mmoles /L of potassium ions,
- (c) from 0.1 to 2.5 mmoles /L of calcium ions,
- (d) from 0.4 to 25 mmoles /L of magnesium ions, and
- (e) from 96 to 126 mmoles /L of chloride ions;

(ii) a buffer component comprising
- (f) from 21 to 27 mmoles /L of bicarbonate ions, and
- (g) from 1 to 12 mmoles /L of TES, MOPS or BES;

(iii) a substrate component comprising:
- (h) 2 to 11 mmoles /L of glucose
- (i) 50 to 150 µmoles /L of glycerol and
- (j) 7 to 15 µmoles /L of choline;

(iv) an amino acid component comprising:
- (k) 5 to 400 µmoles /L of glutamate
- (l) 5 to 200 µmoles /L of aspartate and
- (m) 100 to 2000 µmoles /L of glutamine;

(v) a co-enzyme component comprising:
- (n) 1 to 120 nmoles /L of thiamine cocarboxylase;

(vi) a vitaminoid component comprising:
- (o) 40 to 70 µmoles /L of D- or DL- or L- carnitine;

(vii) a protein component comprising:
- (p) 5 to 200 m I.U./L of porcine or human insulin; and

2. A physiological medium as claimed in Claim 1 which comprises

(viii) an antibiotic component comprising:
(q) 10 to 150 mg/L of chloramphenicol.

3. A physiological medium as claimed in Claim 1 or 2 wherein the salt component comprises:
(c) from 1.0 to 2.5 mmoles/L of calcium ions, and
(d) from 0.4 to 2.4 mmoles/L of magnesium ions.

4. A physiological medium as claimed in any of claims 1 to 3 wherein the salt component comprises
135.32 mmoles /L of sodium ions, 5.00 mmoles /L of potassium ions, 1.25 mmoles /L of calcium ions, 0.45 mmoles /L of magnesium ions, as chloride salts, and 118.40 mmoles /L of chloride ions as sodium, potassium, calcium and magnesium salts.

5. A physiological medium as claimed in any one of Claims 1 to 4 wherein the buffer component comprises 25.00 mmoles /L of bicarbonate ions as sodium salt and 5.0 mmoles /L of N,N-bis (2-hydroxy ethyl)-2- amino-ethanesulfonic acid (BES).

6. A physiological medium as claimed in any of Claims 1 to 5 wherein the substrate component comprises 10 mmoles /L of D-glucose, 110 µmoles /L of glycerol and 10.0 µmoles /L of choline as the chloride salt.

7. A physiological medium claimed in any of Claims 1 to 6 wherein the amino acid component comprises 300 µmoles /L of L-glutamate as sodium salt, 20 µmoles /L of L-aspartate as sodium salt and 400 µmoles /L of L-glutamine.

8. A physiological medium claimed in any of Claims 1 to 7 wherein the co-enzyme component comprises 40.0 nmoles /L of thiamine as thiamine pyrophosphate chloride.

9. A physiological medium as claimed in any one of Claims 1 to 8 wherein the vitaminoid component comprises 50.0 µmoles /L of [-]-β-hydroxy-γ-trimethylaminobutyrate hydrochloride (L-carnitine).

10. A physiological medium as claimed in any one of Claims 1 to 9 wherein the protein component comprises 28.0 m. I.U./L of recombinant human insulin (expressed in E.coli).

11. A physiological medium as claimed in any one of Claims 1 to 10 wherein the antibiotic component comprises 100 mg/L of D-[-]-theo-2-dichloroacetamide-I-(p-nitrophenyl)-1,3-propane acid (chloramphenicol).

12. A method for producing a physiological medium according to any one of Claims 1 to 11 which comprises adding in the following order: sodium chloride, potassium chloride, calcium chloride, magnesium chloride, the TES, MOPS, or BES, thiamine, carnitine, choline, glycerol, insulin, aspartate, glucose, glutamate, glutamine, and sodium bicarbonate to sterile purified water, with constant stirring, making up to the desired volume, filtering and storing in sterile sealed vessels.

13. Concentrates for the preparation of a physiological medium as claimed in any one of Claims 1 to 11 which comprise the salt, buffer, substrate, amino acid, co-enzyme, vitaminoid and protein components, and dilutable with sterile purified water to form said physiological medium.

14. Concentrates for the preparation of a physiological medium as claimed in any one of Claims 1 to 11 which comprise the salt, buffer, substrate, amino acid, co-enzyme, vitaminoid and protein components, except for sodium bicarbonate, and dilutable with sterile purified water with the addition of sodium bicarbonate to form said physiological medium.

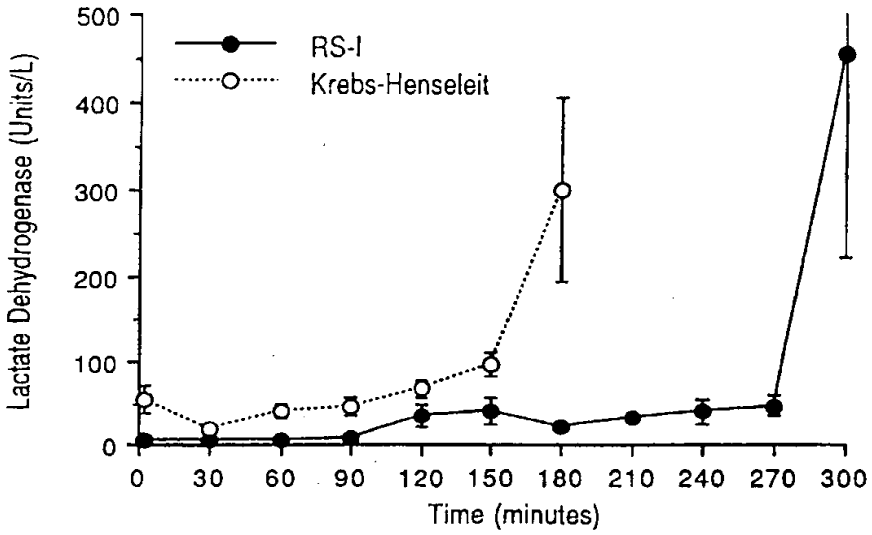
500
400
300
200
100
0
RS-I
Krebs-Henseleit
Lactate Dehydrogenase (Units/L)
0 30 60 90 120 150 180 210 240 270 300
Time (minutes)

Fig. 1

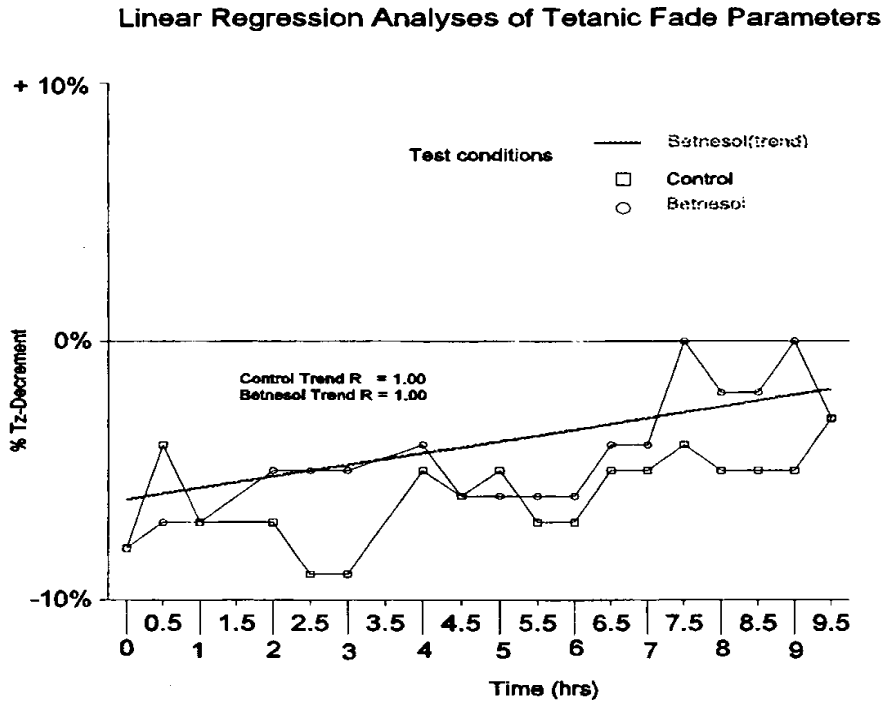

Fig. 2 Analysis of Tetanic Fade Responses over Experimental Time

Fig. 2

Figure 3

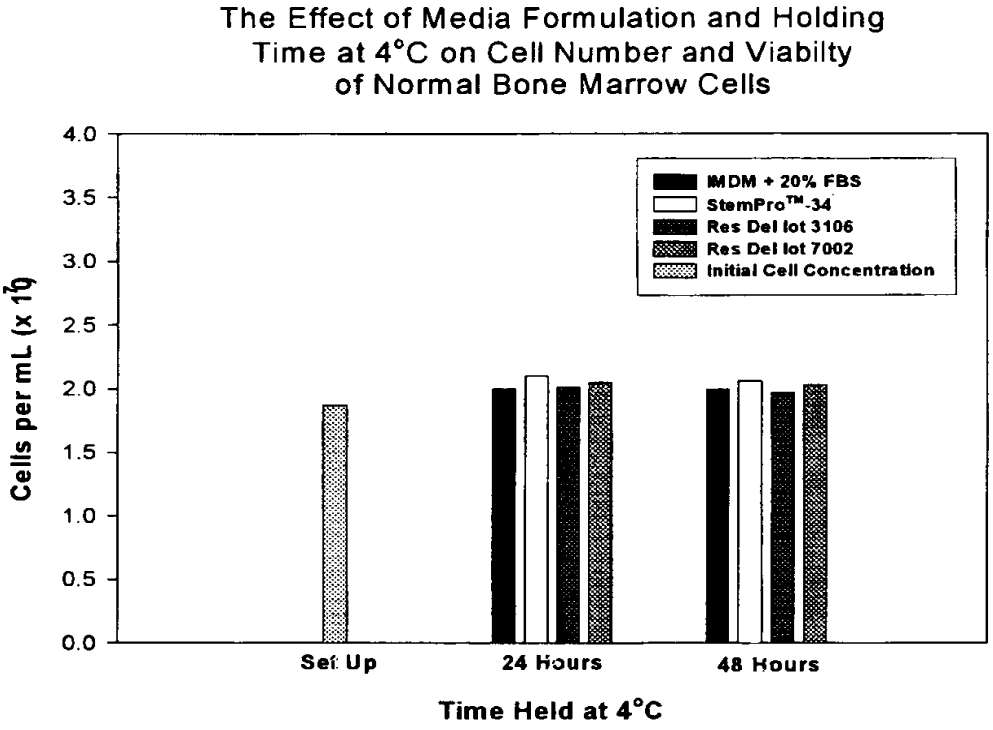

Fig. 3

Figure 4

The Effect of Holding Media and Time on the Ability of Human Bone Marrow Expansion in Culture

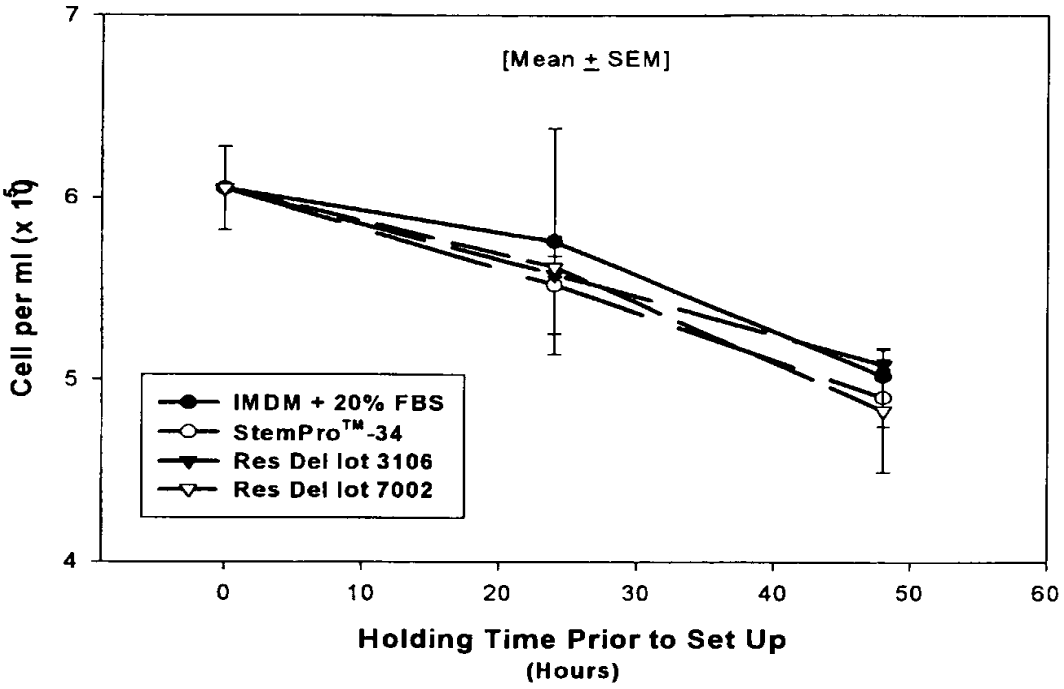

Fig. 4

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) **Title:** PHYSIOLOGICAL MEDIUM FOR PERFUSING, PRESERVING AND STORING ISOLATED CELL, TISSUE AND ORGAN SAMPLES

(57) **Abstract:** A physiological liquid medium is provided having the basic, synergistic components to allow its universal application in preserving cellular and functional integrity *in vitro* of different cell, tissue and organ types isolated from different mammalian species. The medium comprises an aqueous solution in sterile purified water of: i) a salt component comprising: a) from 100 to 150 mmoles/L of sodium ions, b) from 2.5 to 6.2 mmoles/L of potassium ions, c) from 1.0 to 2.5 mmoles/L of calcium ions, d) from 0.4 to 25 mmoles/L of magnesium ions, and e) from 96 to 126 mmoles/L of chloride ions; ii) a buffer component comprising: f) from 21 to 27 mmoles/L of bicarbonate ions, and g) from 1 to 12 mmoles/L of TES, MOPS or BES; iii) a substrate component comprising: h) 2 to 11 mmoles/L of glucose, I) 50 to 150 µmoles/L of glycerol and j)7 zo 15 µmoles/L of choline; iv) an amino acid component comprising: k) 5 to 400 µmoles/L of glutamate, l) 5 to 200 µmoles/L of aspartate and m) 100 to 2000 µmoles/L of glutamine; v) a co-enzyme component comprising: n) 1 to 120 nmoles/L of thiamine cocarboxylase; vi) a vitaminoid component comprising: o) 40 to 70 µmoles/l of D- or DL- or L-carnitine; vii) a protein component comprising: p) 5 to 200 m I.U./L of porcine or human insulin.

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INTERNATIONAL SEARCH REPORT

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A. CLASSIFICATION OF SUBJECT MATTER
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According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, PAJ, WPI Data, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	EP 0 627 161 A (SIGMA TAU IND FARMACEUTI) 7 December 1994 (1994-12-07) the whole document ---	1-14
A	US 5 110 722 A (ANDEREGG KATHERINE A ET AL) 5 May 1992 (1992-05-05) the whole document table II --- -/--	1-14

[X] Further documents are listed in the continuation of box C.

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European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Bertrand, F

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
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